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Environment & Resources DTU
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Preface

The present thesis has been prepared at Environment & Resources DTU, Technical University of Denmark, as part of the fulfillment of the Ph.D. degree requirements. The work was carried out in the period from September 1998 to July 2003 with Associate Professor Niels Nyholm as supervisor and Associate Professor Hans-Jørgen Albrechtsen as co-supervisor. The project was founded by the Technical University of Denmark but received also contributions from the Danish Environmental Research Programme – Pesticides and Groundwater (SMP 96) and from BASF AG, Germany.

The thesis consists of a summary focused on assessing biodegradability in batch simulation test, with specific emphasis on the factors affecting adaptation, and the following five papers (4 published and one manuscript). Since one of the purposes of the summary is to compare the above mentioned papers with the literature, all references to the papers are accentuated with bold. However, the papers are not included in this www-version but can be obtained from the Library at Environment & Ressources DTU, Bygningstorvet, Building 115, Technical University of Denmark, DK-2800 Lyngby (library@er.dtu.dk).

The in-text references and the titles of the papers are:
(Torang et al., 2002); (Torang and Nyholm, 2005); (Ingerslev et al., 2000); (Torang et al., 2003); (Torang et al., 2004).

I Torang,L., Reuschenbach,P., Muller,B., and Nyholm,N., 2002. Laboratory shake flask batch tests can predict field biodegradation of aniline in the Rhine. **Chemosphere, 49: 1257-1265.**

II Torang, L. and Nyholm, N. 2005. Biodegradation rates in adapted surface water can be assessed following a preadaptation period with semi-continuous operation. **Chemosphere, 61: 1-10.**

III Ingerslev,F., Torang,L., and Nyholm,N., 2000. Importance of the Test Volume on the Lag Phase in Biodegradation Studies. **Environ Toxicol Chem, 19: 2443-2447.**

IV Torang, L., Nyholm, N., and Albrechtsen, H. J. 2003. Shifts in biodegradation kinetics of the herbicides MCPP and 2,4-D at low concentrations in aerobic aquifer materials. **Environmental Science and Technology, 37: 3095-3103**.

V Torang, L., Nyholm, N., and Albrechtsen, H. J. 2004. Adaptation behavior during biodegradation of 2,4-D at low concentrations in aerobic aquifer samples. **Draft for Environmental Science and Technology.**

Furthermore two additional papers in English have been published but are not included as part of this PhD thesis:

> Ingerslev,F., Torang,L., Loke,M.L., Halling-Sorensen,B., and Nyholm,N., 2001. Primary biodegradation of veterinary antibiotics in aerobic and anaerobic surface water simulation systems. **Chemosphere, 44:865-872.**

> Toräng,L., Albrechtsen,H.J., and Nyholm,N., 2000. Biodegradation kinetics at low concentrations (<1µg/L) for pesticide contaminants. In: Bjerg, P. L., Engesgaard, P. & Krom, T. D. (eds.): **Groundwater 2000**. Proceedings of the International Conference on Groundwater Research, Copenhagen, 6-8 June, **pp. 167-168.** A.A. Balkema, Rotterdam, NL.

Many individuals were involved in this project which could not have been fulfilled without them. Employees at the section Toxicology and Ecology at BASF AG, Ludwigshafen, Germany, treated me very nice during my visit there. Senior Researcher Udo Pagga and Senior Researcher Peter Reuschenbach should be especially mentioned. The people at Environment and Resources DTU were always professional and ambitious in a pleasant way, providing a strong daily basis for the work. A number of people, both at the department and other institutions, performed different analysis for me once in a while. On a more regular basis Signe Qualmann took samples and generated high quality data for me to work on, and she should be especially thanked. Torben Dolin and Birthe Brejl improved the drawings and Grete Hansen and Helle Offenberg provided literature. During a field trip Bent Skov helped with the hard work. My supervisors, Niels Nyholm and Hans-Jørgen Albrechtsen, were always prepared to read and thoroughly comment manuscripts, and to come up with new ideas for me to work on.

The thesis could not have been written without the understanding, patience, and encouragement of my wife, Signe.

Lars Toräng

Abstract

The aim of this PhD project was to evaluate some of the factors and mechanisms that are responsible for the biodegradation in batch simulation tests with specific focus on adaptation phenomenon and degradation rates.
In **Manuscript I** the aim was to compare degradation rates of aniline in laboratory shake flask simulation tests with field rates in the river Rhine. The combined events of a low flow situation in the Rhine and residual aniline concentrations in the effluent from the BASF treatment plant in Ludwigshafen temporarily higher than normal, made it possible to monitor aniline at trace concentrations in the river water downstream the wastewater outlet by means of a sensitive GC headspace analytical method. Aniline was analyzed along a downstream gradient and the dilution along the gradient was calculated from measurements of conductivity, sulfate and a non-readily biodegradable substance, 1,4-dioxane. Compensating dilution, field first-order degradation rate constants downstream the discharge of BASF were estimated at 1.8 day^{-1} for two different dates with water temperatures of 21.9°C and 14.7°C, respectively. This field rate estimate was compared with results from 38 laboratory shake flask batch tests with Rhine water which averaged 1.5 day^{-1} at 15°C and 2.0 day^{-1} at 20°C. These results indicate that laboratory shake flask batch tests with low concentrations of test substance can be good predictors of degradation rates in natural water bodies - at least as ascertained here for short duration tests with readily degradable compounds among which aniline is a commonly used reference.

Manuscript II describes a *semi-continuous preexposure procedure* (SCEP) for surface water batch simulation biodegradability tests at low chemical concentrations (0.1-100 µg/L). This type of tests are normally used for determining "initial rates" characteristic of the water as sampled, while the aim of the SCEP is the determination of reproducible "adapted rates". The SCEP maintains the microbial diversity and a supply of test substance and natural substrates, and thereby facilitates the process of adaptation. Conducting a SCEP involves regular renewal of a part (e.g. one third) of the test suspension (e.g. every two weeks) using freshly collected natural water with test compound added to the initial concentration. A study prototype was performed with aniline, 4-nitrophenol, 2,4-dichlorophenoxyacetic acid, 4-chloroaniline, and water from the urban river Mølleå. Following preexposure considerably reduced and much more reproducible lag phases resulted, whereas adapted rates of degradation were roughly the same as final rates in batch tests. The adapted rate constant is perceived as an inherent characteristic of the test compound at a specific concentration and under environmental influence (temperature, natural substrates, etc.) but with no simple links back to the original microbial population. By contrast, the initial rates in batch tests are determined also by the microbial population initially present.

In **Manuscript III** it was shown that increasing the total volume of test medium resulted in decreased lag phases in biodegradability shake flask batch tests conducted with either surface water or with synthetic mineral medium inoculated with supernatant from settled activated sludge. Experiments were performed with test volumes ranging from 1.8 ml to 100 L using two ^{14}C-labeled model chemicals, 2,4-D and *p*-nitrophenol both of which are known to be readily degradable after variable lag phases. Lag phases ranged from 2.1 to 30.4 days for PNP and from 16 to 37 days for 2,4-D. Decreasing the test volume tended to increase the lag phase even when a single test batch was redistributed into smaller flasks. At small volumes of 10 ml or less, degradation failed randomly. Our findings are partly explained by the hypotheses that a sufficient total amount as well as a sufficient concentration of specifically degrading microorganisms or consortia of bacteria must be present initially for biodegradation to get

started, from which follows that with too small inoculations or with too small test volumes, biodegradation may fail randomly. A straight forward practical implication of the findings is that the test volume in biodegradability tests can significantly influence the lag time and thus sometimes be decisive for the outcome in biodegradation studies.

The knowledge on degradation kinetic at naturally relevant low concentration is important in order to give qualified estimates for the time needed in order to decrease contaminant concentrations to below the accepted limit of 0.1 µg/L for pesticides. In **Manuscript IV** the biodegradation kinetics of two phenoxy acid herbicides, MCPP and 2,4-D were studied in laboratory batch microcosms at low concentrations (0.025 to 100 µg/L) using ^{14}C-technique with sediments and groundwater from a shallow aerobic sandy aquifer. Below a certain threshold concentration of approximately 1 µg/L for 2,4-D and 10 µg/L for MCPP, the biodegradation followed first order non-growth kinetics and no adaptation was observed within the experimental period of 341 days. Half-lives for ultimate degradation were 500 days for 2,4-D and 1100 days for MCPP at 10°C in unpolluted aquifer sediment, in this environmentally relevant concentration regime. Above the threshold concentrations the biodegradation rate accelerated gradually due to selective growth of specific biomass, which was ascertained from ^{14}C-MPN (most probable number) enumerations of specific phenoxy acid degraders. At the highest concentration tested (100 µg/L), specific degraders increased from 10^{-1} to 10^{5} cells/g during the experiment, and half-lives after adaptation decreased to approximately 5 days. The enhanced rate of degradation by adapted systems was maintained during degradation of the last residuals measured to less than 0.1 µg/L. *In situ* long-term pre-exposure of the aquifer sediment also resulted in significant higher degradation rates of the phenoxy acids.

Adaptation phenomena and biodegradation kinetics of the phenoxy acid herbicide 2,4-D was studied in laboratory microcosms at low concentrations (0.1 to 100 µg/L) using ^{14}C-technique in **Manuscript V**. Experiments were carried out with groundwater amended with 4.3 gSS/L of sediment fines from a shallow aerobic sandy aquifer. Tests were either batch tests or as semi-continuous preexposed tests (SCEP). Conducting a SCEP one third of the test suspension was renewed monthly using freshly collected aquifer material and groundwater with test compound added to the initial concentration. Below a certain threshold concentration of approximately 1 µg/L for 2,4-D, the biodegradation followed first order non-growth kinetics and no adaptation was observed within the experimental period of 335 days. Reproducible half-lives for ultimate degradation were approximately 90 days for 2,4-D at 15°C in unadapted batch test with low spatial variability. At the highest concentration tested (100 µg/L), specific degraders increased by up to a factor of 1000 during the experiment, and half-lives after adaptation decreased to 1-2 days. Adaptation was still prominent 162 d after the batch test and the decay rate was estimated to 0.007 d^{-1}.

In general, the experiments conducted and the literature reviewed suggests that the uses of the standardized laboratory batch simulation tests are necessary and feasible for estimation of biodegradation rates in the environments. However, more knowledge about the complex assessment of biodegradation of organic chemicals at low concentrations in the natural environment is still needed.

Resumé (in Danish)

Formålet med dette PhD projekt har været at undersøge nogle af de faktorer og mekanismer som her betydning for bionedbrydningen i batch simuleringsforsøg med specielt fokus på adaptation og nedbrydnings hastigheder.

I **Artikel I** var formålet at sammenligne nedbrydningshastigheden af anilin i laboratoriet udført som batch simuleringsforsøg i rystekolber med nedbrydningshastigheden bestemt direkte i Rhinen. Et lavt vand flow i Rhinen kombineret med højere anilin koncentrationer end normalt i det rensede spildevand fra BASF´s rensningsanlæg i Ludwigshafen, gjorde det muligt at monitere sporkoncentrationer af anilin i flodvandet nedstrøms spildvandsudløbet med en følsom GC metode. Anilin koncentrationen blev målt over en nedstrøms strækning hvor fortyndingen blev beregnet ud fra målinger af konduktiviteten, sulfat og et ikke let nedbrydeligt kemikalie, 1,4-dioxan. Under hensyntagen til fortyndingen blev den samme første ordens nedbrydningsrate på 1,8 dage^{-1} bestemt på to forskellige dage med vandtemperaturer på henholdsvis 21,9 og 14,7 °C. Denne nedbrydningshastighed blev sammenlignet med 38 batch simuleringsforsøg i rystekolber med vand fra Rhinen og med gennemsnitlige nedbrydningsrater på 1,5 dage^{-1} ved 15°C og 2,0 dage^{-1} ved 20°C. Disse resultater indikerer at batch simuleringsforsøg i rystekolber udført i laboratoriet med lave test koncentrationer kan være gode til forudsigelser af nedbrydningsraten i naturlige miljøer – i hvert fald som undersøgt her i korte tests med let nedbrydelige stoffer som for det ofte anvendte reference stof i nedbrydningsforsøg anilin.

Artikel II beskriver en semi-kontinuer pre-eksponerings procedure (SCEP) for overfladevand til batch simuleringsforsøg i nedbrydningstest med lave koncentrationer (0,1-100 µg/L). Denne type af tests anvendes normalt til at bestemme ”initial rater” i vandprøverne, mens formålet med SCEP er at bestemme reproducerbare ”adapterede rater”. Ved SCEP vedligeholdes den mikrobielle diversitet og en tilførsel af test substrat og naturlige substrater, og skaber derved mulighed for adaptation. Udførelsen af SCEP medfører regelmæssige fornyelse af en del (f.eks. 1/3) af test mediet (f.eks. hver 14.dag) med friskt opsamlet overfladevand tilsat test stoffet til den initiale koncentration. Et test studie med anilin, 4-nitrophenol, 2,4-dichlorophenoxyedikkesyre og 4-chloroanilin blev gennemført med vand fra Mølleåen. Pre-eksponering resulterede i en betydelige reduktion og i meget mere reproducerbare lag faser, mens de adapterede nedbrydnings-hastigheder svarede til dem der blev observeret i slutningen af batch forsøgene. Den adapterede hastighedskonstant opfattes som en iboende egenskab ved test stoffet ved en given koncentration og under påvirkninger fra miljøet (temperatur, naturlige substrater, osv.) med uden nogen simpel sammenhæng til den oprindelige mikrobielle population. Modsat vil initial raterne bestemt i batch forsøg også afhænge af den oprindelige mikrobielle population.

I **Artikel III** blev der ved at forøge det totale volumen af test medium vist reducerede lag tider i batch bionedbrydningsforsøg udført med enten overfladevand eller et syntetisk mineral medium tilsat supernatant fra bundfældet aktiveret slam. Forsøgene blev udført med test volumener på mellem 1,8 mL og 100 L med to ^{14}C-mærkede stoffer, 2,4-D og 4-nitrophenol, som begge er kendt som værende let nedbrydelige efter variable lag faser. Lag faser varierede mellem 2,1 og 30 dage for PNP og fra 16 til 37 dage for 2,4-D. Ved at reducere test volumenet blev forøgede lag faser observeret selv hvis væsken fra den samme flaske blev fordelt til flere mindre volumener. Ved test volumener på mindre end 10 mL udeblev nedbrydningen tilfældigt. Vores observationer kan delvis forklares med hypotesen om at både den totale mængde og koncentration af specifikke nedbrydende mikroorganismer eller

konsortier af bakterier skal være til stede initialt for at bionedbrydningen starter. Heraf følger at man ved for lave podninger eller med for små test volumener risikere tilfældigheder af om nedbrydningen starter. En praktisk betydning af dette er at test volumenet i bionedbrydningstest kan influere på de observerede lag tider og således nogle gange blive afgørende for resultater af nedbrydningsstudiet.

Viden om nedbrydningskinetik ved naturlige lave koncentrationer er vigtige for at kunne give kvalificerede skøn om den tid det tager at reducere en forurening til under grænseværdien på 0.1 µg/L for pesticider. I **Artikel IV** blev nedbrydningskinetikken for de to herbicider, MCPP og 2,4-D, undersøgt i batch forsøg i laboratoriet med sediment og grundvand fra en aerob sandet akvifer ved lave koncentrationer (0.025 to 100 µg/L) og brug af ^{14}C-mærkede stoffer. Under en given tærskelkoncentration på cirka 1 µg/L for 2,4-D og 10 µg/L for MCPP fulgte nedbrydningen første ordens kinetik og adaptation kunne ikke observeres indenfor forsøgsperioden på 341 dage. Halveringstider for ultimativ nedbrydning ved dette miljørelevante koncentration område var 500 dage for 2,4-D og 1100 dage for MCPP ved 10°C i uforurenet akvifer materiale. Over tærskelkoncentrationen accelererede nedbrydningshastigheden gradvis på grund af vækst af specifikke biomasse, hvilket blev eftervist med ^{14}C-MPN tælling af antallet af specifikke phenoxysyre nedbrydere. Ved den højest testede koncentration (100 µg/L), steg antallet af specifikke nedbrydere fra 10^{-1} til 10^{5} celler/g under forsøget, og halveringstiden efter adaptation blev forkortet til cirka 5 dage. De forøgede nedbrydningshastigheder i adapterede systemer blev opretholdt selv ved nedbrydning til koncentrationer under 0,1 µg/L. *In situ* pre-eksponering af sedimentet i akviferen over en længere periode medførte ligeledes i signifikant højere nedbrydningsrater af phenoxysyrerne.

Adaptationsfænomenet og nedbrydningskinetikken af phenoxysyren, 2,4-D blev yderligere undersøgt i en række laboratorium forsøg ved lave koncentration (0,1 til 100 µg/L) med ^{14}C-mærket stof omtalt i **Artikel V**. Eksperimentet blev udført med grundvand tilsat 4.3 gSS/L fine suspenderede partikler fra en aerob sandet akvifer. Forsøgene blev udført både som batch forsøg og som semi-kontinuere pre-eksponerede (SCEP) forsøg. Ved SCEP-forsøget blev en tredjedel af test suspensionen fornyet en gang om måneden med friskt indsamlet akvifer materiale og grundvand tilsat 2,4-D til den initial koncentrationen. Under en given tærskelkoncentration for 2,4-D på cirka 1 µg/L fulgte nedbrydningen første ordens kinetik og adaptation kunne ikke observeres indenfor forsøgsperioden på 335 dage. Reproducerbare halveringstider for ultimativ nedbrydning var cirka 90 dage for 2,4-D ved 15°C i ikke adapterede tests med en lav rummelig variation. Ved den højeste testede koncentration (100 µg/L) forøgedes antallet af specifikke nedbrydere med op til en faktor 1000 under forsøget, og halveringstiden efter adaptation faldt til 1-2 dage. Adaptation i batch testene var stadigvæk udtalt efter 162 dage og en henfaldsrate på 0,007 dage^{-1} blev estimeret.

Generelt viser de udførte laboratorium forsøg og resultater i litteraturen at brug af standardiserede batch simuleringtests er nødvendige og at de gør det muligt at estimere bionedbrydningshastigheder i miljøet. Det er dog nødvendigt at forøge den nuværende viden om bionedbrydning af organiske kemikalier ved lave koncentration for at forbedre den komplekse vurdering af stoffers skæbne i miljøet.

1 Background and aim of the study

Organic chemicals are used in high quantities in modern society throughout the world. About 30,000 different chemicals are marketed in volumes above 1 tonne per year in the European Union (WHITE PAPER, 2001). Chemicals are either destroyed by use and thus producing degradation products, or released into the sewers, soil, surface water, sea, air, or dumped, or incinerated after use. The presence of a high number of chemicals in the environment can be viewed as both a health problem, as an ecotoxicological problem, and as a regulatory problem. Prediction and understanding of the fate of the chemicals are essential to avoid effects on humans and the environment.

The biodegradability of the organic chemicals is one of the major processes determining the concentration in the environment. For assessments of predicted environmental concentrations estimations of degradation rates are needed together with expected releases and route of exposure of the chemicals. To be able to get high quality data performed under similar condition it is important to have a standardized biodegradability test methodology.

The aim of this Ph.D. project has been to increase knowledge and process understanding of biodegradation in batch simulation tests. The objectives of the thesis were: (i) to study degradation mechanisms and phenomenon in batch simulation tests in surface and groundwater, (ii) to investigate effect of the test concentrations, (iii) to investigate other factors affecting the microbial adaptation, and (iv) to investigate whether the standardized simulation tests are adequate for estimation of real world biodegradation rates and how they can be improved.

Thesis delimitations

The thesis has focused on aerobic groundwater and surface water. Generally, the selected model compounds were soluble, with low volatility, and were polar (Log K_{ow}<3) with a limited sorption potential to study the biodegradation process and reduce the effect of partition equilibriums e.g. sorption, bioaccumulation, evaporation, dissolution.

2 Introduction

Degradability is one of the important intrinsic properties of chemical substances that determine their fate and potential environmental hazard. Non-degradable substances will persist in the environment and may consequently have a potential for causing long-term adverse effects on biota. By contrast, degradable substances may be removed in the sewers, in waster water treatment plants or in the environment. Degradation of organic chemicals in the environment influences the exposure and, hence, it is a key parameter for estimating the risk of effects on the biota.

Biodegradation of a chemical substance depends on both the compound specific properties and of the given environment or the given test system. Standardized test systems can be used to produce reproducible results with general information, but improvements of the present standardized test system are necessary as they today often are only used for categorisation of compounds as "readily" and "not readily" biodegradable (Gotvajn and Zagorc-Koncan, 1999). It should in the future be possible to use the test results for quantification and estimation of biodegradation rates. Biodegradation rate estimates are cornerstones in almost any mathematical chemical fate calculation e.g. in risk assessment of chemicals. Directly applicable experimental rate data are however very rarely available, leaving modelers and assessors the option of only educated guesses. As a result of the need for kinetic data, development of standard test guidelines for biodegradability studies in simulation tests with low concentrations of test chemicals have been commenced (see 2.3.3).

There are different ways of assessing the rate of transformation of organic chemicals. The rate can be determined in the field or in the laboratory by assessing the disappearance of the parent compound or accumulation of a final metabolite, at natural environment conditions or in more or less artificial environments. Each procedure has advantages and shortcomings, especially from a modeling perspective. Field measurements are a direct measure of the residual local concentrations under actual conditions and of relevant xenobiotics usage. As such, they must be recommended for monitoring purposes, but field studies are in general very expensive. Yet, there are also several restrictions with respect to the use of field data to feed models. They are obtained in open systems where other dispersion processes (dilution, leaching, volatilization, run off) are not always fully controlled and where additional sinks (photolysis, hydrolysis, and adsorption) contribute making the interpretation of crude

measurements difficult without additional data acquisition in the laboratory. Analytical problems at the low concentrations may also result in either a high data uncertainty or that the field experiments are performed at unrealistic high concentrations. With the exception of persistent chemicals for which long periods of incubation in isolated environments where microorganism may deteriorate in time, laboratory measurements of parent compound disappearance or mineralization have in general to be preferred also from an economical point of view.

The extrapolation of laboratory derived rate constants to the environment requires an understanding of the various biological, chemical, and physical factors and the integration of these effects into mathematical models. The significance of many of the major environmental variables is poorly understood, and few quantitative relationships are available. Several questions regarding the effects of substrate concentration, sorption, oxygen gradients, and the availability of nutrients on biological transformation rates remain to be answered. These effects will need to be evaluated if laboratory data are to be successfully extrapolated to describe the fate of chemicals in the environment (Alexander, 1999;Boethling et al., 1995;Klecka, 1985).

2.1 EU chemical policy

The following section is mainly written based on a report from the European Commission (WHITE PAPER, 2001). The present system in the European Union (EU) for testing general industrial chemicals distinguishes between "existing substances" i.e. the EINECS list with 100,106 chemicals declared to be on the market in September 1981, and "new substances" i.e. some 3,400 new substances placed on the market since that date. Testing and assessing the risks of new substances to human health and the environment according to Directive 93/67/ECC are required before marketing in volumes above 10 kg. For higher volumes more in-depth testing focusing on long-term and chronic effects and degradability has to be provided. By contrast, existing substances amount to more than 99% of the total volume of all substances on the market, and are not subject to the same testing requirements. However, some 140 of these substances have been identified as priority substances and are subject to comprehensive risk assessment carried out by Member State authorities. The risk assessment process is slow and resource-intensive and does not allow the

system to work efficiently and effectively. In recent years, drawbacks of the current system have been identified and examined. The most important of these are:

- 100.106 existing substances can be used without or with few investigations
- burden of proof on public authorities
- no efficient instrument to ensure safe use of the most problematic substances
- lack of incentives for innovation, in particular of less hazardous substitutes

Therefore, the Environment Commissioner Margot Wallström and Enterprise Commissioner Erkki Liikanen both from the European Commission have recently presented a new proposal – REACH - to overhaul and modernize the EU's regulatory system for chemicals which have been in preparation for four years (IP/03/646, 2003). The aims of the proposed new regulation, which will replace 40 different pieces of current legislation, are to increase the protection of human health and the environment from exposure to chemicals while at the same time to maintain and enhance the competitiveness and innovative capability of the EU chemicals industry.

The most important change is that existing and new substances will be assessed in the same way. The “burden of proof” is transferred from the public authorities to the producers / importers. And as a general rule in the new proposed system, information requirements are directly linked to production volumes. Substances produced in amounts of below one tonne per year do not have to be registered. Low volumes have low information requirements.

The system for Registering, Evaluating and Authorizing CHemical products (REACH) should therefore boost enterprise competitiveness and product innovation, to the long-run benefit of chemicals manufacturers and importers, users, consumers and the environment. The general reactions of the proposed REACH system have during an 8 weeks Internet circulation been mixed. Some 6,400 contributions and suggestions were received from public authorities, non governmental organizations (NGOs), industry and individuals. In general enterprises claim that this will damage competitiveness and product innovation and be too expensive with an official estimated total costs of about € 2.1 billion, over the next 9 years until 2012 (WHITE PAPER, 2001). Non government organizations are unsatisfied with REACH because of an increased number of experiments with animals and also that the system in their opinion do not encourage substitution of chemicals and is not strict enough.

Suggested deadlines for submission of registration dossiers are: (WHITE PAPER, 2001):

- substances exceeding a production volume of 1,000 t - at the latest by end of 2004
- substances exceeding a production volume of 100 t - at the latest by end of 2008
- substances exceeding a production volume of 1 t - at the latest by end of 2012

2.2 Risk assessment of chemicals

Any risk assessment on chemicals is composed of two distinct elements (Figure 1), (1) an evaluation of the properties which are intrinsic to the chemical, called *hazard assessment*, and (2) an estimation of the *exposure* which depends on the use of the chemical (Halling-Sorensen et al., 2000). The hazard assessment identifies the inherent *hazardous properties* (e.g. toxicity, carcinogenicity, endocrine disrupting effects, bioaccumulation and ecotoxicity for the aquatic environment) and determines the potency of the chemical with respect to these hazardous properties resulting in an estimation of a Predicted No Effect Concentration (PNEC). The exposure assessment identifies the sources of the chemicals which lead to exposure and estimates the releases of the chemical into a particular compartment of the environment. The importance of exposure has been emphasized in the European "Strategy for a Future Chemicals Policy" (WHITE PAPER, 2001): "Adequate knowledge about exposure is an absolute requirement for any reliable risk assessment". Degradation of organic chemicals in the environment influences the exposure and, hence, it is a key parameter for estimating the risk of long-term adverse effects on biota (Alexander, 1999). Degradation rates, or half-lives, may preferably be determined in simulation biodegradation tests as specified in the Technical Guidance Document (TGD Part II, 2003) conducted with conditions that are realistic for the particular environmental compartment (e.g. surface water, soil, sediment or sewage treatment plant). From the sources and knowledge about degradation (biodegradation, hydrolysis, and photolysis), normally expressed as a combined pseudo-first-order rate constant (TGD Part II, 2003), it is possible to estimate a predicted environmental concentration (PEC).

The risk characterization phase is carried out by determining the PEC/PNEC ratio for all the different relevant environmental compartments both on a local as well as regional scale. As a consequence, the comparison of PNEC values for the different compartments with different PEC values for different exposure scenarios can lead to a number of PEC/PNEC

ratios. When PEC/PNEC ratios greater than one have been calculated, the competent authority should consult industry in order to see if additional data on exposure and/or ecotoxicity can be obtained in order to refine the assessment else risk reduction measures have to be taken (TGD Part II, 2003).

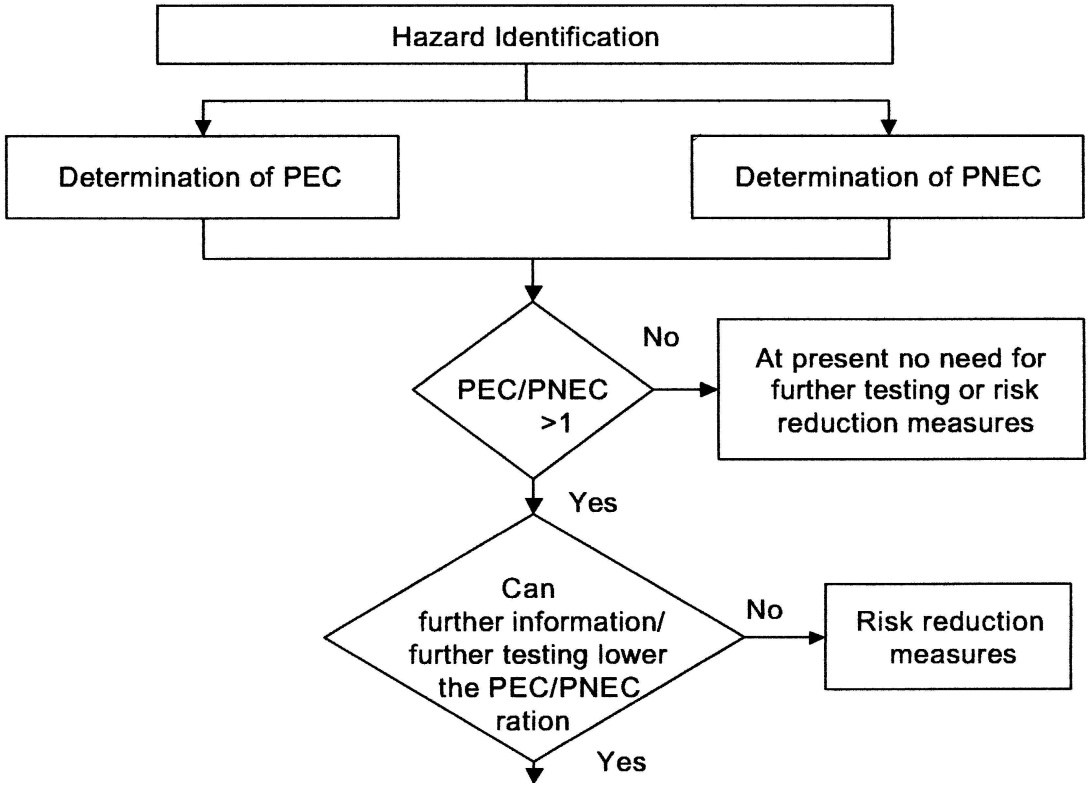

Figure 1. General procedure for chemical risk assessment. Simplified after TGD Part II (2003).

2.3 Biodegradability tests

Standardized biodegradability tests of organic substances are developed by a number of organizations including OECD, ISO, European Commission, US-EPA (OPPTS) and ASTM. The OECD test procedure is used as an example below, but similar test strategies apply for other organizations. An overview of the standardized biodegradability tests can be found in Appendix 2. A tiered examination strategy is applied and the idea behind the test hierarchy is that the relative non-problematic substances are removed at the beginning such that unnecessary testing is avoided (Figure 2). The tests complexity and the economic consequences bound to the tests increases from the simple screening test for ready biodegradability to the more complex simulation tests. Screening tests and are often described

as “acquittal tests”, due to the fact that a positive result causes the examined substance to be classified as “ready biodegradable” (Blok and Balk, 1994). A negative result in a test for ready biodegradability does not necessarily mean that the chemical will not be degraded under relevant environmental conditions, but it means that it should be considered to progress to the next level of testing, i.e. either an inherent biodegradability test or a simulation test. The first option may be used, if data describing the potential biodegradability under optimized aerobic conditions are sufficient for the particular assessment.

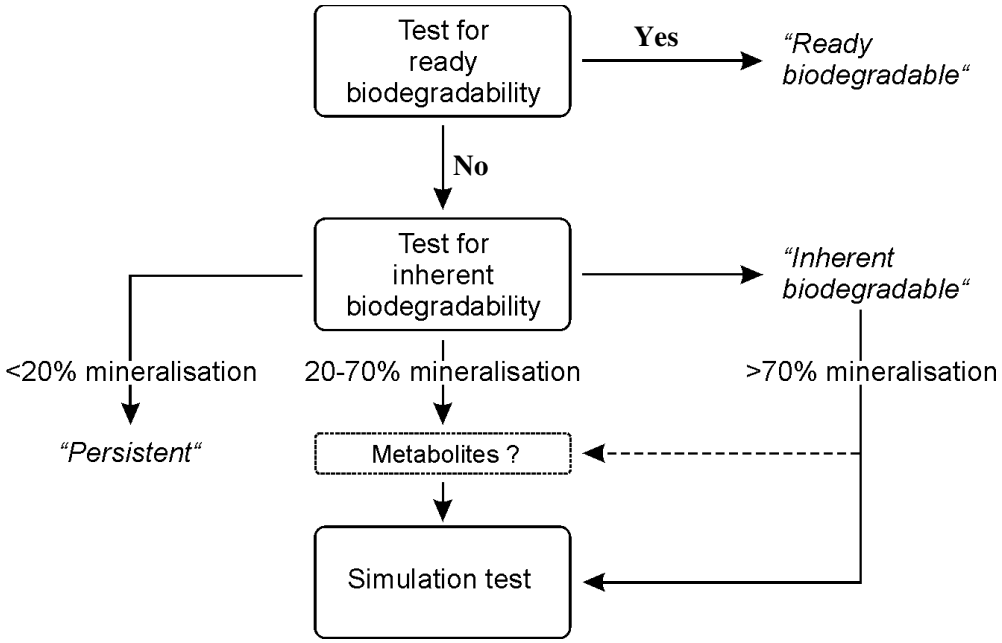

Figure 2. OECD’s strategy for biodegradability testing (modified after Blok and Balk, 1994).

2.3.1. Screening tests

Standard tests for determination of the ready biodegradability of organic substances have been developed by OECD (OECD Test Guidelines 301A-F), EU (C.4 tests), US-EPA / OPPTS (835.3110) and ISO (9408, 9439, 10707). The above test guidelines are similar in several respects. In all the tests, the test substance providing the sole source of organic carbon (except for carbon associated with the inoculum) is diluted in a test medium containing a relatively low concentration of biomass. In all the tests, a non-specific analytical method is used to follow the course of biodegradation. This has the advantage that the methods are applicable to a wide variety of organic substances and there is no need to develop specific

analytical procedures. The ready biodegradability tests are stringent tests, which provide limited opportunity for biodegradation and adaptation to occur. The basic test conditions ensuring these specifications are:

- high concentration of test substance (1-100 mg/L);
- the test substance is the sole carbon and energy source;
- low to medium concentration of inoculum (10^4-10^8 cells/mL);
- no preadaptation of inoculum is allowed;
- test temperature < 25°C;
- 28 days test period with a 10-days time window for degradation to take place; and
- pass levels of 70% (DOC removal) or 60% (O_2 demand or CO_2 evolution) demonstrating complete mineralization (as the remaining carbon of the test substance is assumed to be built into the growing biomass or present as products of biosynthesis).

In these tests, a positive result can be considered as indicative of rapid ultimate degradation in most aerobic environments including biological sewage treatment plants e.g. Struijs and Stoltenkamp (1994). Aerobic ready biodegradability tests are used for aquatic hazard classification of chemicals, and a chemical attaining the pass level in these tests at a certain rate after ended lag phase may be classified as readily biodegradable. Since these methods also respond to any biodegradation residues or transformation products, an indication of the extent of ultimate biodegradation is provided.

2.3.2. Inherent biodegradability tests

Tests which can be used to determine the inherent biodegradability of organic chemicals include e.g. the three methods described in the OECD Test Guidelines No. 302 A-C. Inherent biodegradability tests are designed to assess whether the substance has any potential for biodegradation. Since inherent biodegradability can be considered to be a specific property of a chemical, it is not necessary to define limits on test duration or biodegradation rates. A negative result will normally be taken as an indicator of that non-biodegradability (persistence) should be assumed for precautionary reason. A positive result, on the other hand, indicates that the substance most likely will not persist indefinitely in the environment. The test procedures allow prolonged exposure of the test substance to microorganisms and a low test substance to biomass ratio, which makes the tests powerful. A biodegradation above 20% (measured as BOD, DOC or COD) may be regarded as evidence

of inherent, primary biodegradability, whereas a biodegradation above 70% (measured as BOD, DOC or COD) may be regarded as evidence of inherent, ultimate biodegradability (TGD Part II, 2003). Some of these tests may be conducted using microorganisms that have previously been exposed to the test substance, which frequently results in adaptation leading to a significantly faster degradation of the chemical. Because of the favorable conditions employed in inherent tests, a rapid biodegradation in the environment of these inherently biodegradable chemicals cannot generally be assumed. Extrapolation of the results (TGD Part II, 2003) of the inherent tests should be done with great caution because of the strongly favorable conditions for biodegradation that are present in these tests (see Table 1).

Table 1. First order rate constants and half-lives for biodegradation in surface water based on results of screening tests on biodegradability [a] (after TGD Part II, 2003)

Test result	Rate constant k (d^{-1})	Half-life (d)
Readily biodegradable	4.7×10^{-2}	15
Readily, but failing 10-d window [b]	1.4×10^{-2}	50
Inherently biodegradable [c]	4.7×10^{-3}	150
Not biodegradable	0	∞

Notes to Table 1:

[a] For the use in exposure models half-lives do not need to be corrected for different temperatures. [b] The 10 day time window concept does not apply to the MITI test. The value obtained in a 14-d window is regarded as acceptable in the Closed Bottle method, if the number of bottles that would have been required to evaluate the 10-d window would cause the test to become too unwieldy. [c] The half-life of 150 days reflects a present "best expert judgment".

2.3.3. Simulation tests in water, sediment and soil

Already in the late 70´s the OECD Chemicals Programme identified a need for the use and development of tests that were specific for various environmental compartments and which included measurements of biodegradation kinetics (Nyholm and Torang, 1999). These categories of tests were referred to as simulation tests, and it is noticed that estimation of kinetics already by then was identified as a part of the objective of simulation testing. The actual development of standard simulation tests for regulatory application has been hampered, however, by lack of consensus on principles and insufficient fundamental knowledge on kinetics, but also because higher priority has been given to harmonization and improvement

of test methods at the screening level - the latter tests being more urgently needed for regulatory application.

Simulation tests aim at estimating degradation rates, conducted in a laboratory system with conditions that are realistic for the particular environmental compartment (e.g. surface water, sediment, soil). Simulation tests should mimic the actual environmental conditions such as redox potential, pH, temperature, microbial community, concentration of test substance, and occurrence and concentration of other substrates. These are important factors that determine the environmental degradation of organic chemicals in combination with the intrinsic properties of the chemical (Howard, 1993).

So far the focus has mostly been pointed at the development of inherent tests and especially the screening tests. Less work has been done on the development of simulation tests, but a few tests have recently been adopted. Batch simulation tests to investigate the biodegradation of organic chemicals under environmentally realistic conditions in soil or sediment have been developed: Aerobic and anaerobic transformation in soil (OECD Test Guideline 307); Aerobic and anaerobic transformation in aquatic sediment systems (OECD Test Guideline 308). A simple shake flask batch simulation test with low concentrations of test chemical added to natural surface water have also been developed as a simulator of simple pelagic systems (ISO 14592, 2002;OECD 309 (Revised draft document), 2002).

A low concentration of the test substance is used in the tests designed to determine the biodegradation rate whereas higher concentrations are normally used for identification and quantification of major transformation products. A low concentration of chemical in this type of tests means a concentration (e.g. less than 1 µg/L to 100 µg/L), which is low enough to ensure that the biodegradation kinetics obtained in the test reflect those expected in the environment being simulated (normally first order kinetic is assumed). Typically concentration of some selected organic compounds reported for surface water and for groundwater is shown in

Table 2. Boethling and Alexander (1979) concluded from their experiments, that laboratory test of biodegradation involving chemical concentrations greater than those in nature may not correctly assess the degradation in natural ecosystems and that low substrate concentration can be important in limiting the biodegradation in natural waters. The biodegradation is measured either by ^{14}C-radiolabelling techniques or by specific chemical analyses. Tests of this type may be subdivided according to the environment, which they are designed to

simulate, e.g.: i) soil, ii) aquatic sediments iii) surface water, iv) groundwater, v) seawater, and v) sewage treatment plants.

Because of the importance of biodegradation data to exposure estimations in risk assessment schemes, basic research on biodegradation kinetics in different environments is greatly needed. This should direct the harmonized test guideline development towards more environmentally relevant conditions to provide better estimates of biodegradation rate constants.

The results of simulation tests may include:

- A degradation rate constant (see 3.3)
- Fraction of mineralized ^{14}C, and, if specific analyses are used, the final level of primary degradation
- Identification and concentration of major transformation products. However, screening tests with much higher concentration are often more convenient to use for this kind of studies.
- Check of mass balance

The assessment of biodegradation in surface waters, sediments and soil should, whenever possible, be based on results from tests simulating the conditions in the relevant environmental compartments (see 2.3.3). However, when results from simulation tests are not available and degradation rates are needed for chemical risk assessment on regional scales results from screening tests may be used instead as shown in Table 1 (TGD Part II, 2003).

	Environment	Country	Maximum µg/L	Median µg/L	Reference
Aniline	surface water	Germany	2.4	1.9	(Kußmaul et al., 1975)
	surface water	Holland	12	1.7	(Wegman and De Korte, 1981)
	surface water	Holland	5.8	2.4	(Wegman and De Korte, 1981)
	surface water	Holland	5.5	1.2	(Wegman and De Korte, 1981)
	surface water	Holland	2.4	0.56	(Wegman and De Korte, 1981)
	surface water	Holland	1.6	0.55	(Wegman and De Korte, 1981)
	surface water	Germany	2.4		(Gewässergütebericht, 1987)
	surface water	Germany	1.2		(Kußmaul et al., 1975)
	surface water	USA	9.8		(Jacobson, 1972)
	surface water	Japan		28	(Environment Agency Japan, 1981)
PNP	surface water	Germany	10	2.7	(Rippen et al., 1984)
	surface water	Malaysia	18.8		(Tan and Chong, 1993)
	surface water	Japan	0.13		(Environment Agency Japan, 1981)
	groundwater	Denmark	0.11	0.02	(GEUS, 2001)
	groundwater	Germany	3.80		(Mußmann et al., 1995)
2,4-D	surface water	Germany	0.29		(Europäischen Gemeinschaften,1990)
	surface water	Greece	1.0	0.17	(Albanis, 1992)
	groundwater	Denmark	0.044	0.018	(GEUS, 2001)
	groundwater	Germany	0.30		(BGW, 1987)
	groundwater	Germany	0.10		(BGW, 1987)
MCPP	surface water	Denmark	6.15		(Felding et al., 1995)
	groundwater	Denmark	2.51	0.025	(GEUS, 2001)
4-ClA	surface water	Germany	0.102	0.090	(Gewässergütebericht, 1987)
	surface water	Holland	0.74	0.19	(Wegman and De Korte, 1981)
	surface water	Holland	0.24	0.12	(Wegman and De Korte, 1981)
	surface water	Holland	0.29	0.11	(Wegman and De Korte, 1981)
	surface water	Holland	0.080	0.020	(Wegman and De Korte, 1981)
	surface water	Holland	0.12	0.010	(Wegman and De Korte, 1981)
	surface water	Germany	0.30		(Gewässergütebericht, 1987)
	groundwater	Germany	0.18	0.090	(Kußmaul et al., 1975)
	groundwater	Germany	0.08		(Kußmaul et al., 1975)

Table 2. Concentration of selected model compounds reported for surface water and for groundwater. PNP = p-nitrophenol, 2,4-D = 2,4-dichlorophenoxyacetic acid, MCPP = mecoprop, and 4-ClA = 4-chloroaniline.

3 General aspects of aerobic biodegradation

Natural communities of microorganisms (bacteria, archaea, fungi, and protozoa) in the environment have amazing physiological versatility. They are cable to metabolize and often mineralize an enormous number of organic molecules. Probably every natural product, disregarding its complexity, is degraded by one or another species in some particular environments. If this was not true, such compounds would, this long after the appearance of life on the earth, have accumulated in enormous amounts.

Several conditions must be satisfied for biodegradation to take place in an environment. These have previous been listed by Alexander (1999) and include the following: (i) An organisms that has the necessary enzymes to bring about the biodegradation must exist. (ii) The organism must be present in the same environment containing the chemical. (iii) The chemical must be accessible to the organism having the requisite enzymes. (iv) If the initial enzyme bringing about the degradation is extracellular, the bonds acted upon by that enzyme must be exposed for the catalyst to function. (v) Should the enzymes catalyzing the initial degradation be intracellular, that molecule must penetrate the surface of the cell to the internal sites where the enzymes acts. (vi) Because the population or biomass of bacteria or fungi acting on many xenobiotics is initially small, conditions in the environment must be conducive to allow for proliferation of the potentially active microorganisms.

3.1 Definition of biodegradation

Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemicals. Organic chemicals may undergo primary biodegradation resulting in alteration of the chemical structure of a substance brought about by biological action. Ultimate biodegradability under aerobic condition is the breakdown of an organic compound to CO_2, water, the oxides or mineral salts of other elements, and/or to products associated with normal metabolic processes of microorganisms (See Figure 3). Finally, if the test substance is fully mineralized this will result in a complete oxidization to CO_2, H_2O and minerals salts.

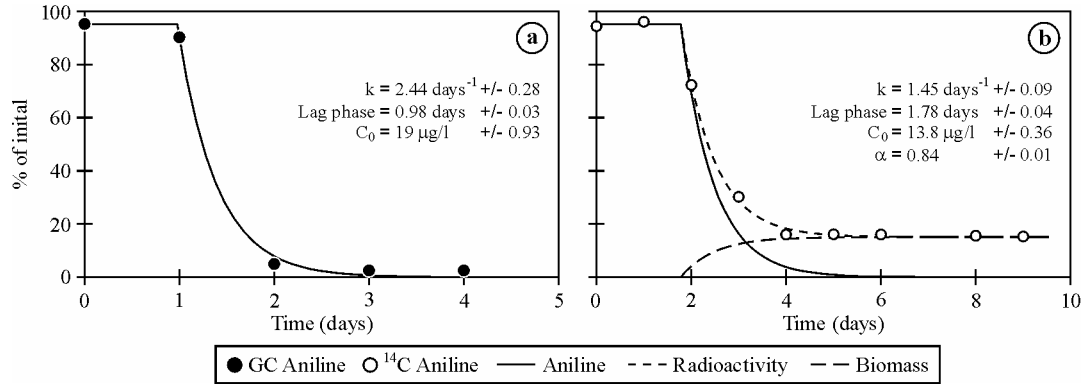

Figure 3. Example of degradation curves for aniline from batch experiments using a) GC headspace (primary degradation), test temperature: 20°C. Example of experiment with (a) problematically low number of data points. (b) Measurements obtained with ^{14}C techniques (Ultimate biodegradation), test temperature: 15°C. +/-: estimated standard deviation. From Torang et al., 2002.

3.2 Degradation of organic chemicals in batch simulation tests and in the natural environment

Biodegradation in surface water and groundwater is depending on a number of factors including conditions in the particular environmental compartment, the chemical substance, and the active microbial populations. The importance and the relevance of these factors in batch simulation tests and in natural environments are shortly discussed in the following sections.

3.2.1. Physical, chemical factors in the environmental compartments

Factors such as **temperature, pH, nutrients and redox potential** have high importance to the survival and growth of microorganisms, and therefore these factors are also very important to the degradation of organic chemicals. The conditions found in the environment can to some extent be maintained in batch simulation tests, and the estimated rates in the laboratory tests can thus be supposed to reflect the degradation rates in the field.

Surface water characteristics. In Danish rivers the yearly average temperature are 8.5°C but with seasonal variations from 0 to 25°C (Andersen et al., 2002). The pelagic surface water is normally aerobic and the pH is generally between 6 and 9 (Severinsen et al., 1996). The hydraulic retention times is determining whether biodegradation can be a significant removal process. In most Danish rivers the retention time is less than 1 day, whereas the 25th, 50th, and 75th percentiles were 25, 140, and 390 days in 204 greater Danish

lakes (mainly bigger than 5 ha) (Jensen et al., 1997). Surface water from the lowland river MølleÅ near Copenhagen used in this study receives water from an eutrophic lake and is free from wastewater discharge, but the river receives occasional overflows from sewers during rainstorms. In the river Mølle Å the yearly average values (mg/L) in 2001 were BOD_5 = 4.58, NH_4-N = 0.11, NO_3-N = 0.55, total N = 1.56, orto-phosphate = 0.06, and total P = 0.20 (NOVA-2003, 2003). The total numbers of microorganisms present in surface water vary considerably typically in the range of 10^3-10^7 cells/mL (Ingerslev and Nyholm, 2000;**Torang and Nyholm, 2005**);Lehmicke, 1979 334 /id;Kuenemann, 1992 224 /id}.

Aquifer characteristics. The temperature in shallow Danish aquifers is typically in the range from 8-12°C and the pH is generally between 5 and 8 (Bjerg and Christensen, 1992;Pedersen, 2000;**Torang et al., 2004**). Oxygen is usually present in the upper layers of uncontaminated aquifers due to the infiltration of aerobic rainwater. Other electron acceptors often take over with increasing depth but this is not considered in the present thesis (for details see e.g. Appelo and Postma (1993) and Pedersen (2000). The content of total organic carbon is normally low in pristine sandy aquifers and is found either as sediment bound carbon with fractions of 0.01-0.48% or in the groundwater with 1-12 mgC/L (Christensen et al., 1996;Pedersen et al., 1991;Pedersen, 2000). The content of both organic and inorganic nutrients are low and the groundwater environment can often be characterized as oligotrophic. The numbers of microorganisms present in pristine aquifers vary considerably (10^2-10^4 cells/mL in the groundwater and 10^3-10^6 cells/g sediment) (Albrechtsen and Winding, 1992;Dobbins et al., 1992;Ghiorse and WILSON, 1988), but are in general orders of magnitude lower than in top soil. The majority of microbial biomass in aquifers is found attached to sediment phase normally in the range from 97-100% (Albrechtsen, 1994;Harvey et al., 1984).

Geology and sediment composition are also significant to chemical and biological processes in the aquifers. The distribution of clay, silt, sand and gravel influence characters as pore volume, porosity and permeability which on the other hand determine the accessibility of water and air to the groundwater and thereby also of e.g. nutrients and oxygen. The grain size, mineralogy, pH and organic carbon content influences the sorption characters of a soil and thus also the degradation of xenobiotics (Madsen et al., 2000;Mihelcic et al., 1993;Scow and Johnson, 1997).

3.2.2. Factors determined by the xenobiotic compound

The **chemical structure** of xenobiotic compound has a profound effect on degradation. Even for compounds with high structural similarities, significant differences in their degradability exist. As an example, a higher degradation rate for 2,4-D compared to other phenoxy acids herbicides is typically found, and 2,4,5-trichlorophenoxyacetic acid is usually degraded slowest (Mccall et al., 1981;Smith and Aubin, 1991;**Torang et al., 2003**;Zipper et al., 1999).

A number of chemical equilibrium reactions are important for the fate and in which phase a chemical compound is present, e.g. **sorption, dissociation, evaporation, dissolution, ion-exchange, complex formation** (for details see Schwarzenbach et al. (2003)). These equilibrium reactions are very important for both the possibilities for biodegradation to occur and to whether a compound will be transported to the atmosphere, the sea, or to the groundwater. To study the biodegradation process and reduce effect of the other partition equilibriums, the selected model compounds were as a general soluble, with a low volatility, and were polar (Log K_{ow}<3) with a limited sorption potential. Aniline, 4-nitrophenol (PNP), 2,4-dichlorophenoxyacetic acid (2,4-D), mecoprop (MCPP) and 4-chloroaniline (4-ClA) were selected as model compounds covering a range of degradabilities from easily biodegradable to recalcitrant (see Figure 4).

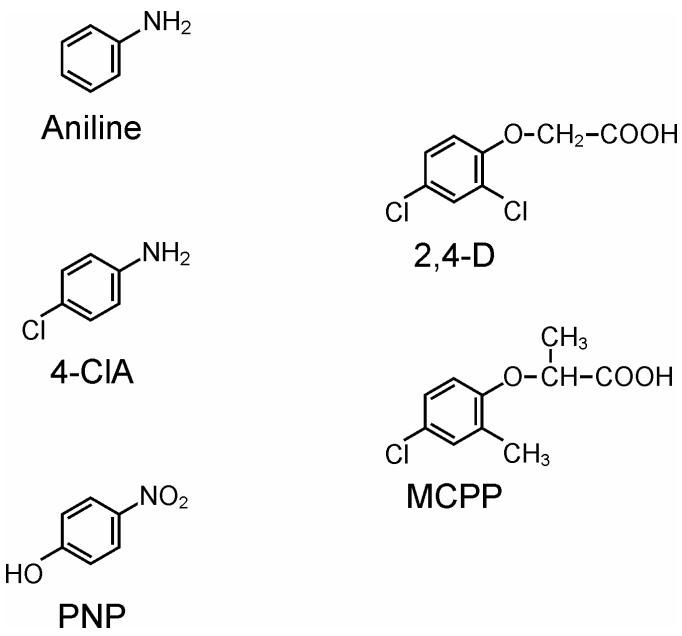

Figure 4. Molecular structure of aniline, 4-chloroaniline (4-ClA), p-nitrophenol (PNP), 2,4-dichlorophenoxyacetic acid (2,4-D), and mecoprop (MCPP).

3.2.3. Biological factors

Prior to the degradation of many organic compounds, a period is noted in which no destruction of the chemical is evident. This time interval is designated as an adaptation period or lag phase and can be considered to end at the onset of the period of detectable biodegradation. During this interval, no change in the concentration is noted, but then the disappearance becomes evident and the rate of degradation often becomes more rapid.

Factors affecting adaptation. Many explanations have been proposed for the adaptation of microbial communities to the biodegradation of organic compounds in surface water or groundwater. These explanations are related to (i) proliferation of small populations; (ii) presence of toxins; (iii) predation by protozoa; (iv) appearance of new genotypes; (v) induction of enzymes; and (vi) diauxie (Alexander, 1999;Wiggins et al., 1987). Few of the explanations, however, were derived from studies of natural microbial communities acting on xenobiotics at environmentally relevant concentrations, and hence the original emphasis placed on certain of these hypotheses must be considered with skepticism.

The duration of the adaptation period is not fixed but varies from site to site, and some microbial communities adapt to a particular chemical whereas others do not. Such variation in the occurrence of adaptation has been noted for 4-nitrophenol added to fresh and marine waters (Spain and Van Veld, 1983) and 2,4-D added to surface water (Hoover et al., 1986;**Torang and Nyholm, 2005**). Lag phases are poorly reproducible and can last for weeks or months (Alexander, 1999;Ingerslev and Nyholm, 2000;Nyholm et al., 1984;Nyholm et al., 1992;Nyholm and Torang, 1999;Painter, 1995;Spain, 1990;**Torang and Nyholm, 2005**), and this variable duration makes planning of sampling for kinetic experiments difficult.
Limitations of the test systems. Small test volumes can also be a cause of longer and less reproducible lag phases in aquatic biodegradation tests. Increased lag phases or failure of degradation were observed for PNP and 2,4-D in experiments with test volumes of less than 50 mL (Figure 5). Painter (1995) have earlier stated that biodegradation tests should be inoculated with cell densities of least 10^4 cells/mL (corresponding to a total of $2 \cdot 10^6$ cells in a 200 mL test solution) as a minimum to avoid the risk that the concentration of competent microorganisms becomes critically low and also to avoid variability between replicates. In top soil tests significant differences between replicates based on 16S rDNA analysis have been observed when the sample size was less than 10 g (Ellingsoe and Johnsen, 2002). False negative test results may also occur due to the circumstance that the simple batch system used has a limited lifetime and may deteriorate in time and loose its specific degradation ability and/or its similarity with nature (Ingerslev and Nyholm, 2000). False negative results mean

failure of compounds to degrade in the specific test system even though general environmental degradability has been established.

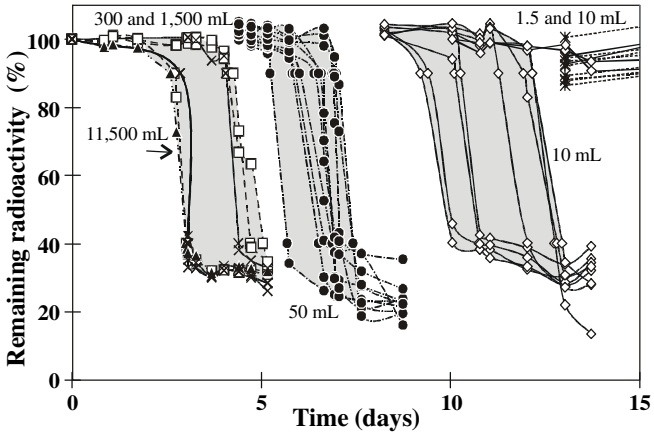

Figure 5. Biodegradation of 5 mg/L p-nitrophenol in synthetic medium inoculated with 50 ml/L activated sludge supernatant. Redistribution of a large batch into smaller volumes of (numbers of replicates indicated in parentheses): 1.5(10) [-∗-], 10(10) [-◇-], 50(10) [-●-], 300(4) [-□-], 1,500(3) [-x-], and 11,500(1) [- ▲-] ml. From Ingerslev et al., 2000.

To mitigate the problems of poorly reproducible lag phases in batch simulation tests, preadaptation of the inoculum to low tests concentrations has been investigated in surface water (Ingerslev and Nyholm, 2000;**Torang and Nyholm, 2005**) and groundwater (**Torang et al., 2004**). The aim was to eliminate the risk of false negative test results and to minimize the problem of variable duration of lag phases and thus optimize sampling schemes. The procedure is carried out in practice by periodically renewing part of the test suspension with freshly collected surface water making up the replaced volume of water with test compound to the starting concentration, and in this way maintaining the system characteristics (see Figure 6). This resulted in an elimination or reduction in the lag phases without increasing the subsequent degradation rates other than marginally (**Torang and Nyholm, 2005**). However, the use of a semi-continuous preexposure procedure (SCEP) seem to result in adaptation at 1 µg/L for 2,4-D, which is lower than observed for batch tests (**Torang et al., 2004**). Possible explanations include the input of new primary substrates resulting in less decay and the higher total exposure of the test system with the test substance compared to the batch tests.

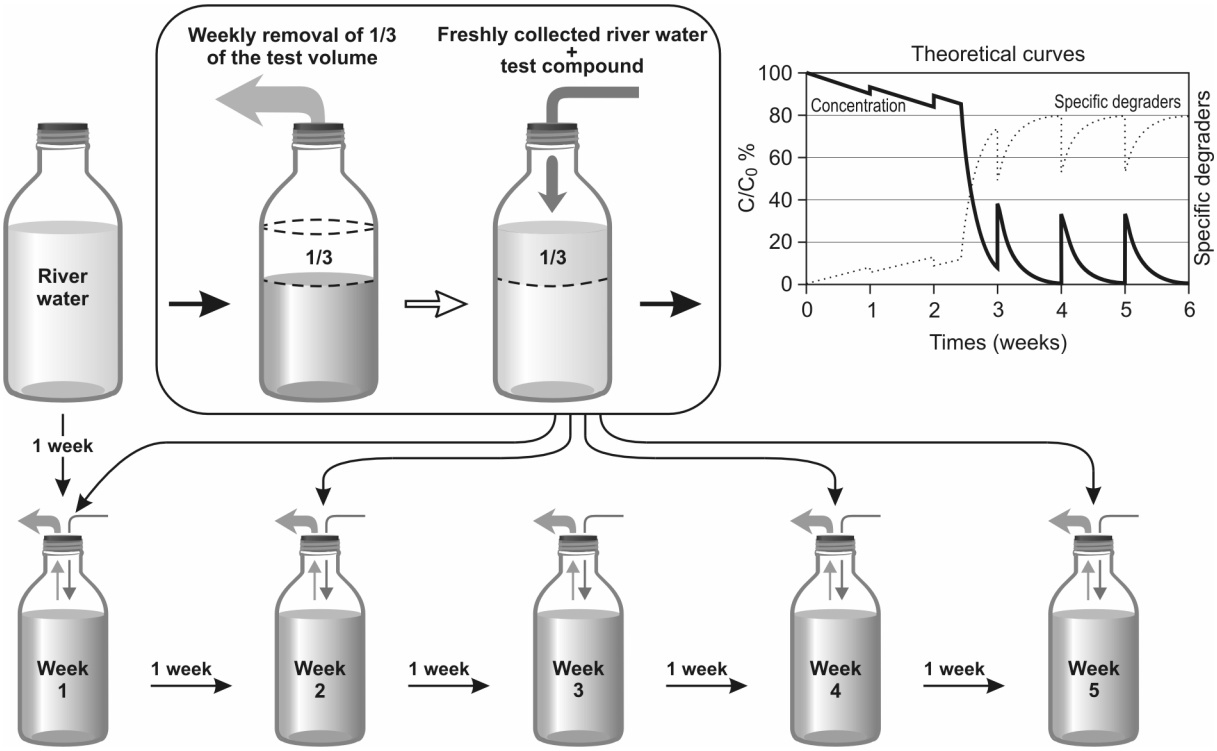

Figure 6. A semi-continuous preexposure procedure (SCEP) with low concentrations of test compound. One third of the water volume is renewed each week. From Torang and Nyholm, 2005.

Detection and quantification of specific degraders are needed to determine whether growth of specific degraders takes place. Different techniques can be used for detection, quantification and characterization of the microbial population degrading the test substance. Organisms with special physiological or catabolic characters can be measured by using e.g. selective agar plates, most probable number (MPN) techniques, or different types of molecular techniques (Skipper et al., 1996). Good correlation have e.g. been observed between most probable numbers of 2,4-D degraders and the gene probes, *tfdA* (monooxygenase) and *tfdB* (hydroxylase), but poor agreement were observed with four other gene probes for enzymes also associated with biodegradation of 2,4-D (Holben et al., 1992). However, several bacterial 2,4-D degraders have been reported that do not share homology with the *tfd* genes of strain JMP134 (Cavalca et al., 1999;de Lipthay et al., 2003;Fulthorpe et al., 1995;Kamagata et al., 1997;Vallaeys et al., 1996). Even though that problem with underestimation of the degrading population may also be present with the MPN method, especially if the microorganisms are agglomerated in flocks or biofilm, this method was

selected in the present study because the same procedure for estimation of specific degraders could be used for all the different model compounds (**Torang et al., 2003**).

Threshold concentration for growth. There appear to be concentrations of some chemicals below which no adaptation occurs and where also the biodegradation kinetics shifts (See Figure 7). Below a certain threshold concentration, typically < 10 µg/L, no appreciable growth of specific degrader organisms can be detected which often result in true first order degradation kinetics (see 3.3.2). This contrasts with growth-linked kinetics with accelerating degradation rates, which are often observed at higher concentrations (Alexander, 1999;Reynolds et al., 1991). A few reports have been published on concentration-dependent shifts in biodegradation kinetics, where the degradation curves indicated shifts from growth-linked kinetics with biphasic curves to non-growth kinetics. Examples of threshold concentrations for various environmental compartments include: groundwater (Aelion et al., 1987;Aelion et al., 1989;**Torang et al., 2003**), sewage (Rubin et al., 1982), salt marsh cores (Spain and Van Veld, 1983), lake water (Hoover et al., 1986;Subba-Rao et al., 1982) and river water (Boethling and Alexander, 1979;Ingerslev and Nyholm, 2000). Subba-Rao et al., (1982) found that the degree of mineralization was high below the threshold concentration where more than 98% of aniline, 94% of benzoate, 96% of phenol, 97% of benzylamine and 94% of 2,4-D were mineralized suggesting that organic compounds may be degraded without incorporation of carbon into cellular components.

A theoretical model based on thermodynamic and diffusion has been developed for estimating the threshold concentration of an organic compound required to support the multiplication of a bacterium and predict a minimum substrate concentration of 0.2 µg/L (Schmidt et al., 1985a). Below this concentration organisms would either suffer from a negative growth rate or a shrinking cell size and continued metabolism of the substrate would not occur. The thresholds can be interpreted as the concentration levels where the net growth of specific biomass is zero (loss equals total growth). This theoretical approach only applies for pure cultures and single substrates and can not necessarily be extended to the real world. With concurrent utilization of natural substrates this level can be hypothesized to depend on the specific substrate as well as the bioavailable carbon (**Torang et al., 2003**).

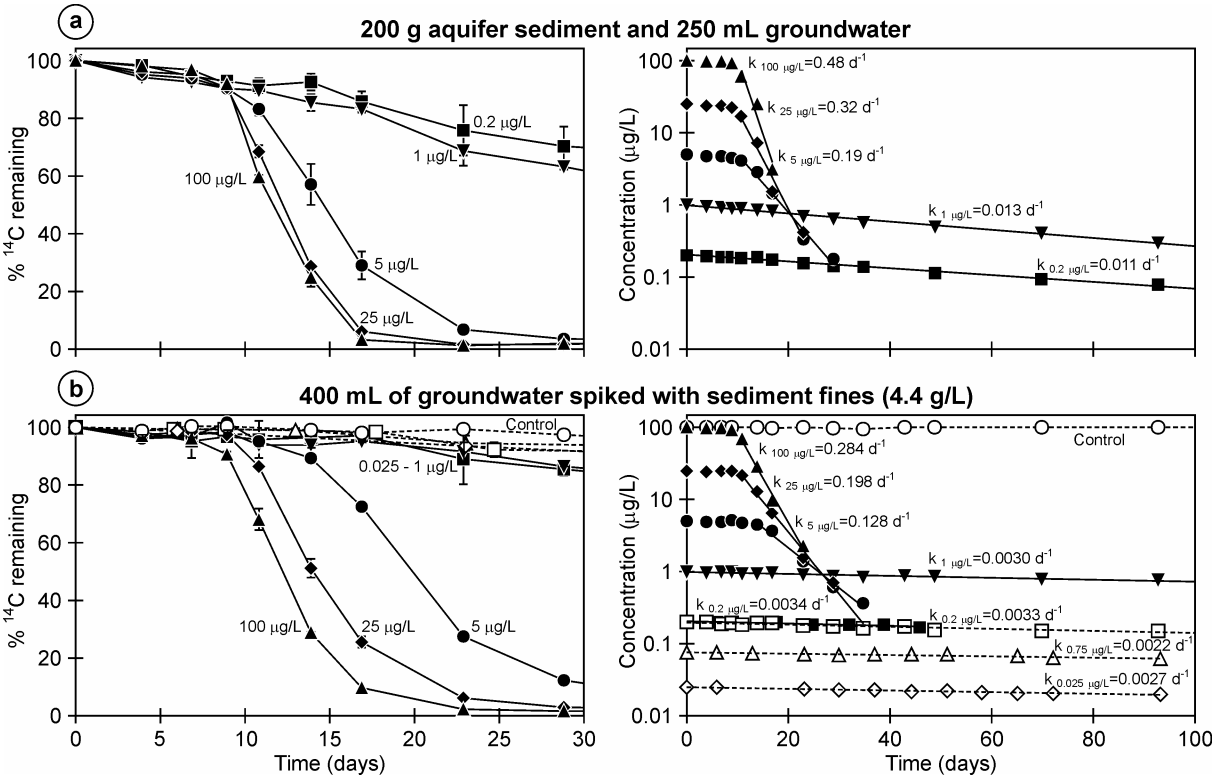

Figure 7. Degradation of 2,4-D at different concentrations, under aerobic conditions, at 15°C, in (a) aquifer sediment + groundwater; (b) groundwater spiked with aquifer sediment fines, from a sandy unpolluted aquifer near Vejen, Denmark. Means and standard deviations were based on data from duplicate flasks and data are shown in % ^{14}C remaining, and the initial concentrations are given on the left graph. To the right the data are given as concentrations (logarithmically transformed) and modeled with simple first order kinetics. From Torang et al., 2003.

The threshold phenomenon has also been studied in pure cultures. (Pahm and Alexander, 1993) studied the effect of a low concentrations of p-nitrophenol (PNP) on growth of four PNP-degrading bacteria and their abilities to metabolize low concentrations of the compound in culture and samples from an oligotrophic lake. PNP did not increase the growth rates of *Flavobacterium sp. M4, Pseudomonas sp. K, Flavobacterium sp. M1,* and *Pseudomonas sp. SP3* at concentrations of less than 2, 4, 10, and 100 µg/L, respectively, when it was the sole added carbon source in culture, but it stimulated multiplication at higher concentrations. By contrast, when each of the bacteria was separately inoculated into samples of water from an oligotrophic lake and from a well in which PNP was not biodegraded, the bacteria were able to mineralize as little as 1 µg PNP/L. The addition to a salts solution of 10 µg of glucose per L resulted in mineralization of PNP at concentrations too low to be mineralized when the nitro compound was the sole source of added carbon. Bacteria may thus be able to mineralize substrates in natural waters at concentrations below those suggested by

tests conducted in culture media, possibly because of the availability of other carbon sources for the bacteria (Pahm and Alexander, 1993).

Utilization of mixtures of carbon sources. Many environments contain levels of organic C in excess of that needed to support growth, or the levels may be regenerated constantly by excretions of other organisms or by new inputs. Under these conditions, the energy needs for maintenance and growth of the microbial populations degrading the compounds of interest may be met by use of other organic molecules. Microorganisms may metabolize two, or sometimes more, organic substances simultaneously provided that their concentrations are not excessively high (Alexander, 1999;Egli, 1995). The compound sustaining growth and is present at levels above the threshold are often called the *primary substrate*, and the compound that is below the threshold but is still degraded has been designated the *secondary substrate* (Rittmann, 1985). With concurrent utilization of natural substrates threshold level can be hypothesized to depend on the specific substrate as well as the bioavailable carbon.

There is some experimental evidence that carbon starvation or slow growth in carbon-limited continuous culture provokes the expression of many carbon catabolic enzyme systems, although the appropriate carbon sources are absent, resulting in cells that are able to immediately utilize these carbon substrates if they become available in the environment (Kovárová-Kovar and Egli, 1998;**Torang et al., 2003** - see Figure 8). Studies by different research groups have shown that under such conditions heterotrophic microorganisms do not restrict themselves to the utilization of a single carbon source but are simultaneously assimilating many of the compounds available in their environment, even mixtures of carbon sources that normally provide diauxic growth at high concentrations (for detailed review see Egli (1995)). So the potential to utilize different carbon sources simultaneously has to be taken into account when considering microbial competition at low environmental concentrations (Schmidt and Alexander, 1985). This can maybe also explain why compounds are utilized at concentrations at which they will no longer support growth on their own (i.e., below their threshold concentration for growth).

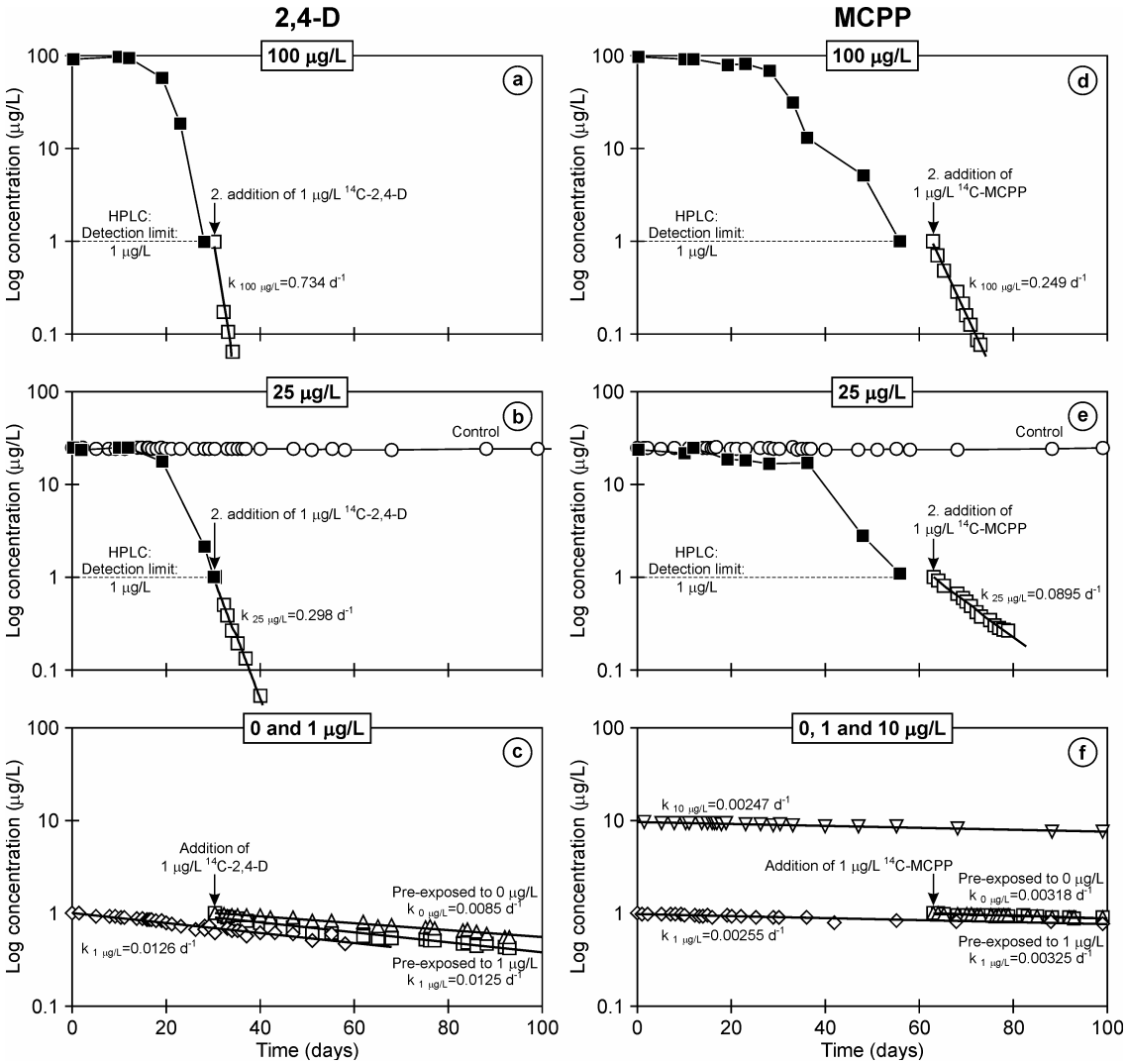

Figure 8. Re-addition of 1 µg/L ^{14}C-labeled phenoxy acid to batch test systems, previously exposed to different concentrations: (a) 2,4-D, 100 µg/L (b) 2,4-D, 25 µg/L; (c) 2,4-D, 0 and 1 µg/L; (d) MCPP, 100 µg/L; (e) MCPP, 25 µg/L; (f) MCPP, 0, 1 and 10 µg/L. Experiments were performed at 10°C, under aerobic conditions, and with low pre-exposed aquifer sediment. From Torang et al., 2003.

3.3 Degradation kinetics

Biodegradation rates are not intrinsic properties for chemicals. Degradation kinetics are complex and for a given chemical within any ecosystem, rate and extent of degradation will possible be subject to daily, seasonal, and spatial variations. The rate of biodegradation in surface water, soil, groundwater and sediment is related to several parameters as discussed above. An accurate estimate of the rate of biodegradation is thus difficult even if laboratory or field data are available.

Several kinetic models have been proposed in the last fifty years and good comprehensive reviews of biodegradation kinetics are available (see e.g. Alexander (1999;Battersby (1990); Kovárová-Kovar and Egli (1998);Mills et al. (1982);Reynolds et al. (1991)).

3.3.1. Monod kinetics

One of the most commonly used models is based on the early work by Monod (1949). Although the Monod model was developed for pure cultures of bacteria growing on a single substrate, it also provides a good approximation with growth of mixed cultures (Simkins and Alexander, 1984).

The specific growth rate follows from Monod (1949):

$$\mu = \mu_{\max} \cdot \frac{S}{S + K_s} \qquad \textbf{Equation 1}$$

where μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (μg/L), and K_s is the substrate saturation constant (μg/L).

If cometabolism is neglected and it is assumed that biodegradation processes are directly related to microbial growth, the overall equation for the degradation process is:

$$\frac{dS}{dt} = -\frac{\mu \cdot B}{Y} \qquad \textbf{Equation 2}$$

where Y is the yield coefficient (μg of biomass/μg of test substance) which is assumed to remain constant during degradation, and B is the specific biomass (μg/L)

Hence the disappearance of substrate may be represented by:

$$\frac{dS}{dt} = -\frac{\mu_{\max} \cdot B \cdot S}{Y \cdot (S + K_s)} = \left(-\frac{\mu_{\max} \cdot X \cdot S}{S + K_s}\right) \qquad \textbf{Equation 3}$$

This is the generally accepted form of the Monod model. Measuring B can be difficult (it refers to the number of specific degraders) but it is not needed for determination of μ_{max} and

K_s and so B/Y may be replaced by X, the amount of substrate needed to produce a population density of B.

In a batch system it may at any time be assumed, that the sum of the concentration of the substrate and B/Y is a constant. This is a conservation equation, where the subscript $_0$ means, concentration at time zero, and can be used if the cell decay process is neglected.

$$S + X = S_0 + X_0 \qquad \textbf{Equation 4}$$

Combining Equation 3 and equation 4 results in:

$$\frac{dS}{dt} = -\frac{\mu_{max} \cdot (S_0 + X_0 - S) \cdot S}{S + K_s} \qquad \textbf{Equation 5}$$

Simkins and Alexander (1984) used this general kinetic model to describe the relationship between growth rate and substrate concentration where the growth dynamics were limited only by the concentration of one substrate (metabolic degradation) as a basis. They developed for batch systems under a range of specific conditions with different substrate and biomass ratios six simplified models describing mineralization kinetics: zero-order, Monod without growth, first-order, logistic, Monod with growth, and logarithmic as illustrated on Figure 9. However, the Monod model with growth has too many parameters to be estimated in a batch experiment without correlation between parameters (Fomsgaard, 1997). Development of kinetics models have been continued by Alexander and his group including 12 models to describe the metabolism of organic substances that are not supporting growth, either because the degradation is cometabolic or because the substrate of interest is present at a very low concentration and therefore not important in determining the growth rate of the active specific degraders (Schmidt et al., 1985b). With no growth and low concentration of test substance the theoretical considerations also result in a first-order model.

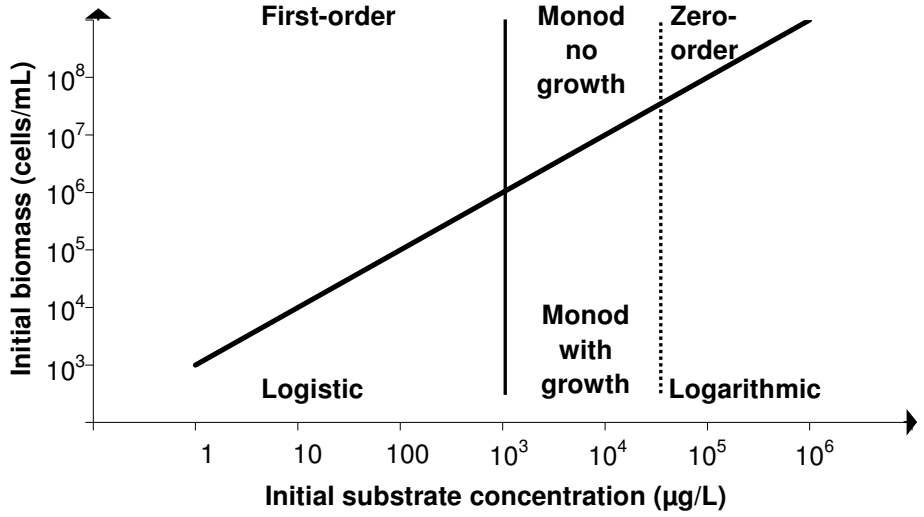

Figure 9. Applicability of six kinetic models as a function of initial substrate concentrations and cell density). Conceptual figure based on degradation experiments with benzoate and a pure culture of *Pseudomonas sp.* Zero order: $-dS/dt=k_0$ (if $X_0 >> S_0$ and $S_0 >> K_s$); Monod, no growth: $-dS/dt=k_0 \cdot S/(K_s+S)$ (if $X_0 >> S_0$); First-order: $-dS/dt = k_1 \cdot S$ (if $X_0 >> S_0$ and $S_0 << K_s$); Logistic: $-dS/dt=k_2 \cdot S \cdot (S_0+X_0-S)$ (if $S_0 << K_s$); Monod with growth: $-dS/dt=\mu_{max} \cdot S \cdot (S_0+X_0-S)/(K_s+S)$ (no limitations); Logarithmic: $-dS/dt = \mu_{max} \cdot (S_0+X_0-S)$ (if $S_0 >> K_s$). Modified after Alexander (1985).

Viewed from a mathematically point of view K_s and μ_{max} are highly correlated parameters (Mølgaard, 1992), and often can in fact only their ratio can be estimated in batch experiments with confidence (see e.g. Grady et al. (1996);Liu and Zachara (2001);Magbanua et al. (1998);Tuxen et al. (2002) for further details). For mixed cultures, it can be expected that the kinetic parameters, and in particular the saturation constant, K_s, are even more variable than with pure cultures (Alexander, 1999). In tests with soil or biofilms diffusion resistance may cause further variability. For these reasons the "Monod equation" is indeed often quite impractical.

3.3.2. First-order kinetics

At the low concentration of test substances relevant in batch simulation tests, degradation curves have most frequently been modeled with first-order kinetics. If the concentration of the test substance, $S << K_s$, e.g. less than 100 µg/L, the Monod equation may be simplified and the degradation rate expressed with first order kinetics.

$$\frac{dS}{dt} = -k_1 \cdot S \qquad \textbf{Equation 6}$$

or in the integrated form:

$$S = S_0 \cdot \exp^{-k_1 \cdot t} \qquad \textbf{Equation 7}$$

When degradation follows first-order kinetics, the half-life of the substance will be independent of the concentration and can be calculated as:

$$T_{½} = \frac{\ln 2}{k} \qquad \textbf{Equation 8}$$

The first-order model is not only easy to use, but as discussed previously (Boethling and Alexander, 1979;Larson and Cowan, 1995;Nyholm and Torang, 1999;Shimp et al., 1990;Simkins and Alexander, 1984), it is often valid at environmentally realistic conditions with low concentration of test substance and biomass.

3.3.3. Pseudo-first-order kinetics

Fate and exposure models usually assume the simplifications, that the kinetics of biodegradation are pseudo-first order and that only the dissolved portion of the substance is available for biodegradation. For a variety of organic chemicals, biodegradation can be described by a simple pseudo-first-order model in which the biodegradation rate is directly proportional to the chemical concentration. The model is regarded as pseudo-first-order due to the contribution of microbial density on degradation rates (Paris et al., 1981;Paris and Rogers, 1986). That is, the resulting pseudo-first-order rate constant is a multiple of the specific microbial density in an environment and an “intrinsic” second-order rate constant. The applicability of the pseudo-first-order model to biodegradation processes has been suggested for different chemicals, particular at environmentally relevant chemical concentrations (Larson, 1984;Lewis and Gattie, 1991). The biodegradation rate will thus directly reflect the fluctuations in size of the degrading community, which is expected to depend mainly on the amount of other easily available carbon substrate.

In this thesis the pseudo-first-order kinetic is used assuming that the concentration of biomass (B) is approximately constant after adaptation during the period with observable degradation (see **Torang and Nyholm (2005**) for detailed discussion). This is convenient because it allows a direct comparing of the pseudo-first-order rate constants with first-order rate constants.

$$\frac{dS}{dt} = -k_2 \cdot B \cdot S = -k_{adapted} \cdot S \qquad \textbf{Equation 9}$$

where k_2 is a second-order rate constant ($L \cdot \mu g^{-1} \cdot day^{-1}$), B is the specific biomass (µg/L), S is the test concentration (µg/L), and $k_{adapted}$ is the pseudo-first-order rate constant after adaptation (day^{-1})

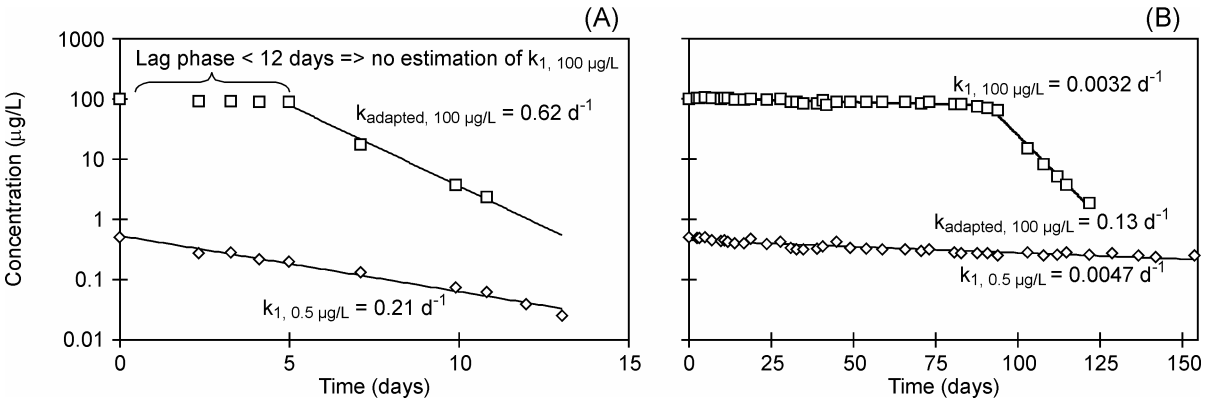

Figure 10. An example of data-analysis from simple one step batch experiments with (a) aniline and (b) 4-chloroaniline at 0.5 and 100 µg/L. At the highest concentration, with growth linked degradation, adaptation took place, and in some tests both an initial and an adapted rate constant could be determined. First-order biodegradation rate constants are estimated by regression on log-transformed concentrations. From Torang and Nyholm, 2005.

3.3.4. Simple interpretation of biodegradation kinetic

A kinetic laboratory batch test may potentially provide two conceptually different rate constants: 1) a rate constant characterizing the water investigated in its sampled state and estimated as an initial rate, and 2) if adaptation takes place, a second rate constant for adapted water may be derived from the last part of the degradation curve (See Figure 10 or Nyholm and Torang, 1999 for further details). If a constant rate has been achieved the water can be regarded as fully adapted. The role of adaptation may vary from being little to being dominating and depends on the properties of the chemical as well as on the test conditions. Typically, initial or immediate rates are obtained from batch tests with freshly collected or freshly inoculated microbial reagent. Such rates characterize the test environment in its non-perturbed state – pristine or naturally exposed. The rates are influenced by the initial

microbial population, if the biomass/chemical concentration ratio is large enough to sustain a significant degradation in itself. Rates in systems that have fully adapted, on the other hand, characterize a steady-state with respect to loading (exposure) and degradation (after sufficient time also a batch test may reveal such conditions). Normally, adapted rates bear no relationship to the original microbial population, because a new population develops as a specific response to the imposed conditions including the exposure to the chemical. Sometimes the time needed to reach full adaptation can be very long (several months or even years) while in other situations adaptation may be completed within a few days, or even less than a day (Alexander, 1999).

4 Fate of selected model compounds in batch simulation tests

A range of degradation rate constants was found for each test substance in our batch simulation tests and in the literature. This is not surprisingly because of the different water samples, test concentrations, and temperatures used. Nevertheless general conclusions about the biodegradability behavior that was consistent with a priori knowledge of the compounds could be drawn.

4.1 Surface water

4.1.1. Aniline

Aniline is well known as a ready biodegradable compound and has been used as a reference compound in regulatory testing (for an overview see Painter (1995)). The adaptation in laboratory batch test to degrade aniline normally takes place fast during a short lag phase of 0 – 7 days (Nyholm and Torang, 1999). Subba-Rao et al. (1982) reported mineralization rates corresponding to half-lives of approximately 3 days for aniline at concentrations from 0.0057 to 5 µg/L in water from Lake White, NY, USA. In our laboratory studies with concentrations less than 10 µg/L degradation half-lives range in freshly collected surface water from 0.4 days in the river Rhine (example shown in Figure 11) to average half-lives of 2.0 days in laboratory tests with surface water the river Mølleå (**Torang et al., 2002**;**Torang and Nyholm, 2005**). In two studies by Ingerslev and Nyholm (2000) and Ingerslev et al. (2001), also with surface water from the river MølleÅ, half-lives for aniline ranged 10-14 days in the summer time while longer half-lives of 15-33 days were observed in the winter time. The lower degradation potential during winter time have also been reported in a study by Osaki et al. (1991) where longer lag phases and slightly slower biodegradation rates of aniline were observed in winter compared to summer under identical laboratory conditions. A lower activity and/or number of microorganisms at winter times possibly explain the longer adaptation period under winter conditions (Gocke and Rheinheimer, 1988).

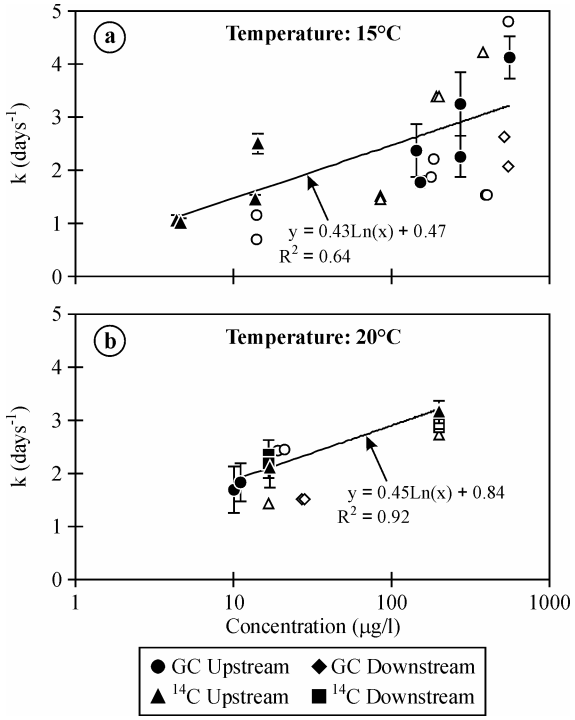

Figure 11. First-order rate constants estimated for aniline in batch simulation tests with Rhine water sampled up and downstream the biological treatment plant of the BASF. Test temperatures 15°C (a) and 20°C (b). Open patterns indicate high uncertainty due to too few data points within the degradation time window. Error bars indicate the estimated standard deviation. From Torang et al., 2002.

Much slower degradation rates have been observed in oligotrophic humic rich water from the small forest creek Fønstrup Bæk, Denmark with half-lives as long as 92 days (Ingerslev and Nyholm, 2000). Half-lives as long as 150 days have been reported in oligotrophic Finnish lake water with high content of humic acids (Ahtiainen et al., 2003). The slow degradation rate of aniline have here been explained by the pristine environment with low numbers of specific degraders as much faster degradation was observed in the urban harbor area of Helsinki. One might also speculate that sorption to the high concentration of humic substances may reduce the bioavailability of aniline. Sequential extraction of sediment treated with ^{14}C-labeled aniline has suggested that aniline is bound through cation-exchange and covalent binding processes, and with longer reaction periods sorption became increasingly dominated by covalent binding. The reaction kinetics for the slow, irreversible sorption of aniline appeared to be limited by the reactivity and/or availability of covalent binding sites (Weber et al., 2001).

Results from an ISO ring test (Nyholm and Torang, 1999) of the biodegradability of aniline clearly demonstrated that batch simulation test with pelagic surface water is a suitable

way to quantitatively investigate biodegradation rates at low test concentrations. However, these results also illustrate the high variability which can and must be expected in batch simulation tests as seen on Figure 12. Correlation between the degradation rate constants and initial concentration of aniline was evident here and higher pseudo first order rates constant were estimated at 100-600 µg/L than at lower concentrations from 1-19 µg/L. It has earlier been reported that aniline degradation can be positively correlated with the extent of water pollution (Osaki et al., 1991), and one can speculate that the major cause of a high aniline degradation rates is the loading of the surface water with e.g. domestic and industrial wastewater causing exposure to a variety of chemical substances, including aniline and structurally related compounds.

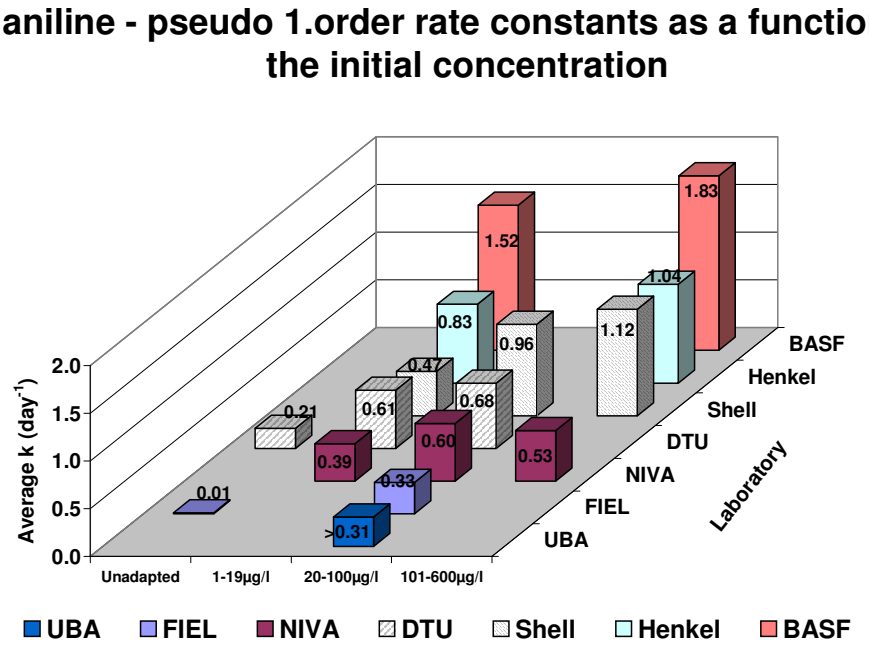

Figure 12. Average degradation rate constants for aniline in an ISO ring test from each participant grouped for different concentration intervals. From Nyholm and Torang, 1999.

Field observation of degradation rates of aniline have been estimated and reported a few times. (Zoeteman et al., 1980) accomplished a monitoring study with Rhine water in the Netherlands of a wide range of chemicals. They collected samples in July 1979 from two locations separated by a distance corresponding to 24.5 hours travel time for water and used the measured concentrations to calculate a half-life for the compounds assuming no important

discharge in between. Their field observation included an estimation of an aniline half-life of 2.3 days or a factor 6 higher than observed in than observed by **Torang et al. (2002**). Teichmann et al. (2002) used the data from **Torang et al. (2002**) together with two additional data sets for ammonia and conductivity in a 2D-model developed for the simulation of mass transport and degradation of substances in the river Rhine and their estimations of the half-life of 0.4 days for aniline are in excellent agreement with the results found with the simple 1D-model (**Torang et al., 2002**) (Figure 13).

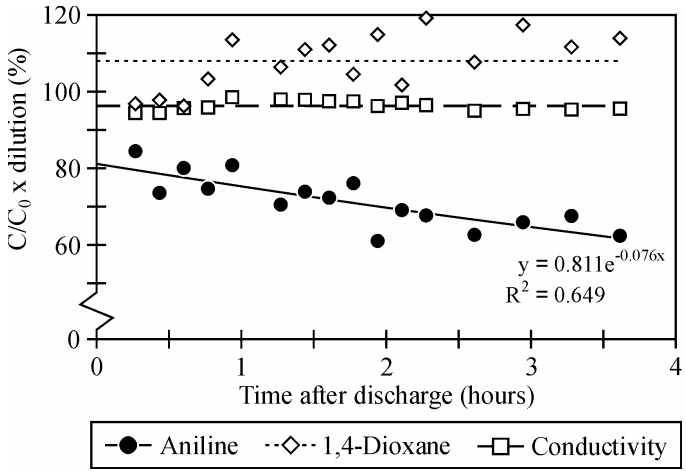

Figure 13. Normalized concentration of aniline, 1,4-dioxane and conductivity in the Rhine downstream the WWTP of BASF, Ludwigshafen as a function of time after discharge (flow rate = 0.83 m/s). All concentrations are corrected for dilution as estimated from sulfate concentrations. From Torang et al., 2002.

4.1.2. PNP

4-nitrophenol (PNP) is a frequently used model compound in biodegradation studies and the compound is ready biodegradable after a highly variable lag phase depending on the initial inoculum (for an overview see Alexander (1999;Painter (1995;Wiggins et al. (1987)). Lag phases of 1.7 to 30 days have been reported for tests with surface water at test concentration below 2 mg/L (Hoover et al., 1986;**Ingerslev et al., 2000**;Spain and Van Veld, 1983;**Torang and Nyholm, 2005**;Wiggins et al., 1987;Wiggins and Alexander, 1988). In a series of papers (Spain et al., 1980;Spain et al., 1984;Spain and Van Veld, 1983), Spain et al. studied adaptation phenomenon and biodegradation of PNP under field condition in ponds. They concluded that the results of exposure in laboratory tests fairly well reflected the results in the field sites. For example, the lag phase in the pond treated with PNP was 130 hours

compared with 110 hours in a flask test with sediment, 88 hours in ecocores and 160-170 hours in large and small microcosms in the laboratory. Such information increases the confidence that the time required for adaptation and subsequent biodegradation of at least some pollutants can be predicted from appropriate laboratory tests. In a detailed process mechanisms study by Wiggins et al. (1987), it was concluded that the time for enzyme induction, mutation, diauxie, and the presence of toxins were not the causes of the lag phases for PNP mineralization in aquatic environments but rather that the lag phase largely reflected the time for multiplication of the initial small population of active organisms. Gericke and Rheinheimer (1991) reported that PNP during summer normally were biodegraded rapidly in the freshwater area of the Elbe River. By contrast, degradation of PNP decreased significantly during periods of low temperature or low oxygen content.

Subba-Rao et al. (1982) reported mineralization rates corresponding to half-lives of 10 to 30 days for PNP at concentrations from 0.0006 to 200 µg/L in samples of Cayuga and Beebe Lake (NY, USA) water, while Ingerslev and Nyholm (2000) estimated half-lives of 16-36 days in the river MølleÅ. In our laboratory studies with concentrations less than 2 µg/L degradation half-lives range from 2 to 5 days in freshly collected surface water from the river MølleÅ (**Torang et al., 2002**;**Torang and Nyholm, 2005**). Kalsch et al. (1999) did some laboratory simulation tests with both natural water and sediment to study the biodegradability of low concentration of chemicals in surface waters by measuring the mineralization and fate of 0.37 µg kg^{-1} [^{14}C]-4-nitrophenol. During 35 days of incubation, non-reducing conditions prevailed in the test reactors and the overall microbial activity did not vary substantially. Half-lives of 17-39 days were estimated for PNP but the test system does not allow separating the degradation in water and the sediment phase.

4.1.3. 2,4-D

2,4-dichlorophenoxyacetic acid (2,4-D) have been widely used as a herbicide and are among the chemicals for which an adaptation period is often observed (Alexander, 1999). Long and highly variable lag phases ranging from 0 to 71 days have been reported in surface water (Boethling and Alexander, 1979;Ingerslev and Nyholm, 2000;**Torang and Nyholm, 2005**).

Subba-Rao et al. (1982) reported mineralization rates of 2,4-D corresponding to half-lives of 15 to 30 days at concentrations from 0.0015 to 5 µg/L in water from lake Beebe (NY,

USA). Ingerslev and Nyholm (2000) estimated half-lives of 23-347 days in the concentration range from 0.2 to 500 µg/L. In surface water adapted to low concentrations (<10 µg/L) of 2,4-D biodegradation proceeded with half-lifes in the range from 0.7 to 13 days (**Torang and Nyholm, 2005**). However, degradation of 0.5 µg/L of 2,4-D was much more slowly in freshly collected river water with half-lifes of 180 days (range: 90-290) and here adaptation could not be recognized based on the degradation curves (Figure 14b) (**Torang and Nyholm, 2005**).

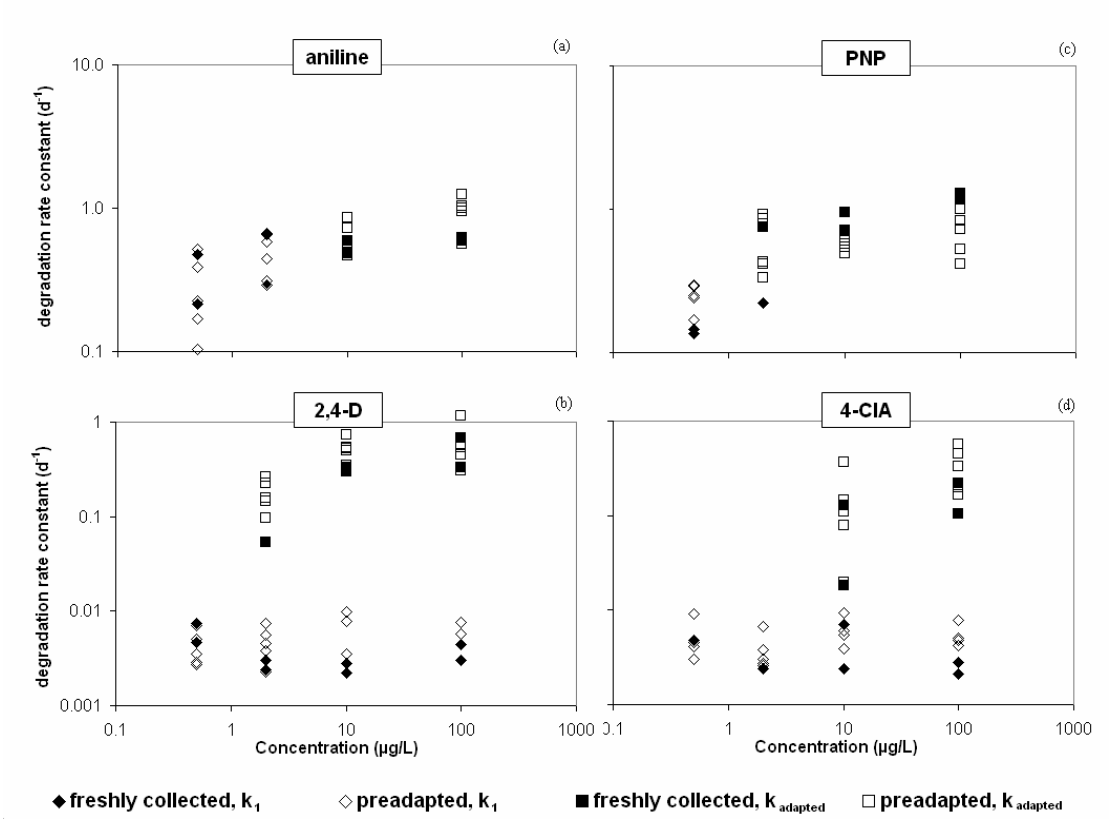

Figure 14. Degradation rates in biodegradation tests with different chemical concentrations and exposure history (freshly collected river water or preadapted river water). Rates of biodegradation were expressed either as first-order rate constants, k_1 or as pseudo-first-order rate constants, $k_{adapted}$ for adapted test systems. From Torang and Nyholm, 2005.

4.1.4. 4-chloroaniline

Torang and Nyholm (2005) studied the biodegradation rates of 4-chloroaniline (4-ClA) in batch simulation tests and considerable variations in lag phases were observed in the range from 22 to 88 days while the degradation half-lives were approximately 220 days (range: 150-290). However a notable exception was seen one time with the freshly collected

river water where degradation of 4-ClA suddenly occurred rapidly with a half-life of only 4 d at the two lowest concentrations tested (0.5 and 2 µg/L). Similar inconsistency in biodegradation half-life, with more recalcitrant compounds have earlier been observed by Ingerslev and Nyholm (2000) with estimated half-lives for 4-ClA from 50 to 347 days in the river MølleÅ. Ahtiainen (2002) reported half-lives for 4-ClA in the range of 70 to >300 days at concentrations from 2 to 26 µg/L with water from two Finish lakes.

The batch simulation test procedure has been evaluated in a ring-test organized by ISO with 4-ClA as one of the model compounds (Nyholm and Torang, 1999). Figure 15 shows results with 4-chloroaniline for the different participating laboratories grouped into rate constants for non adapted systems and adapted systems, respectively. Here, the non adapted first order rate constants ranged from 0.002 to 0.013 d^{-1} (half-lives from 340 to 54 days) with distinct differences amounting to a factor of 6 between the lowest and the highest result. In average the non adapted first order degradation rate equalled 0.006 d^{-1} ($t_{½}$ = 120 days). Adapted rate constants range from 0.08 to 0.29 d^{-1} ($t_{½}$ from 9 to 2.4 days) with an average of the laboratory means of 0.2 d^{-1}. It is evident that adaptation plays an important role for the degradation behaviour of 4-chloroaniline as previously discussed by Kuiper and Hanstveit (1984). It was also noticed that adaptation can take place randomly among replicate flasks (Nyholm and Torang, 1999). There were rather large differences among the individual results reported by each participant, which follows from the random nature of adaptation. Overall, the rate data collected for especially the adapted systems are nevertheless relatively consistent.

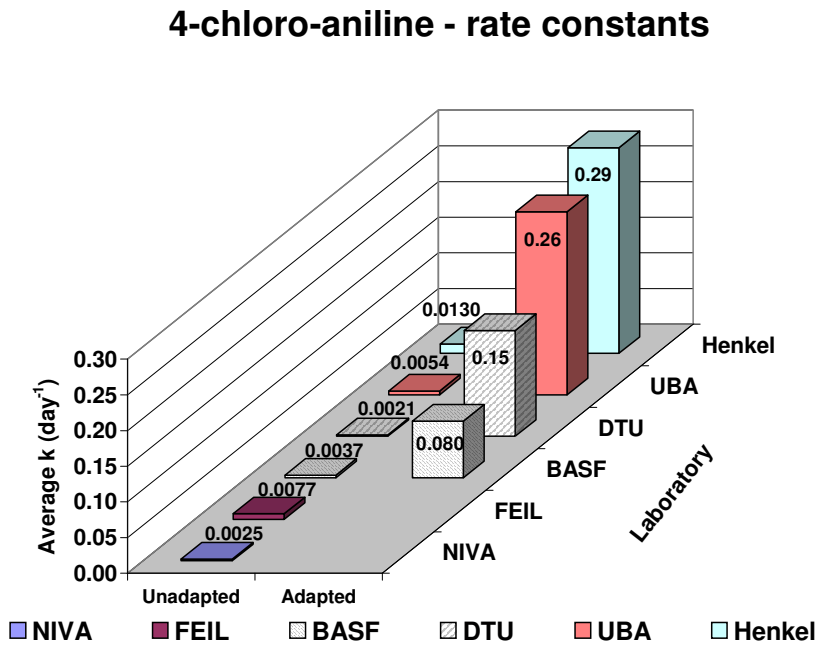

Figure 15. Ring-test results with 4-chloroaniline. Rate constant averages from each participant laboratory grouped for either adapted or non adapted tests. From Nyholm and Torang, 1999.

4.2 Groundwater

Pesticide contamination of groundwater is an example of a well documented and extensive pollution problem characterized by low chemical concentrations (Barbash et al., 2001;Kolpin et al., 1998;Scheidleder et al., 1999). The problem is of high regulatory concern in Europe because of widespread use of groundwater for drinking water and frequent occurrences of concentrations of individual pesticide above the European drinking water standards of 0.1 µg/L (EEC, 1980). No standardized batch simulation tests are available for groundwater and limited knowledge exist about the degradation process at the low pesticide concentrations (typically up to a few µg/L, (GEUS, 2001)). However, a batch simulation tests for groundwater should mimic the conditions e.g. pH, temperature, nutrients, redox and the processes taking place e.g. sorption, abiotic degradation, biodegradation. The tests should also limit significant changes in the water to sediment ratio, but for sampling reasons more water is often added. One might also consider including the transport of water, substrates and microorganisms in the tests to simulate the natural groundwater flow, however this will significantly increase the work load and the complexity of the test system and are therefore not suitable for standard simulation batch tests. If a higher degree of realisms is needed than it is possible to create in simple batch tests e.g. with respect to the groundwater flow, it should

be considered to run continuous column experiments (see e.g. Tuxen et al. (2000)). Rügge et al. (2000) concluded for six pesticides including MCPP, that laboratory batch and column experiments are reliable test systems since results in these systems showed a good accordance with the results obtained in a field injection experiment.

Several investigations have shown the potential for aerobic degradation in groundwater environments of the phenoxy acids MCPP (Agertved et al., 1992;Broholm et al., 2001;Heron and Christensen, 1992;Klint et al., 1993;Larsen et al., 2000;Larsen and Aamand, 2001;Rügge et al., 2000;**Torang et al., 2003**;**Torang et al., 2004**;Tuxen et al., 2002), and 2,4-D (Kuhlmann et al., 1995;Rügge et al., 2000;**Torang et al., 2003**;**Torang et al., 2004**;Tuxen et al., 2002). By contrast, Pedersen (2000) found only limited degradation potential within 370 days of MCPP in laboratory batch incubations with groundwater and sediment material from two aerobic aquifers. Similarly, MCPP and 2,4-D were only degraded in some of some of the batch incubations with material from an aerobic sandy aquifer (Tuxen et al., 2002), and MCPP was not degraded within a period of 200 days in samples from a chalk aquifer (Johnson et al., 2000). Most of these studies mentioned above were conducted with pesticide concentrations higher than 25µg/L (often due to analytical limitations), and extrapolation of the observed degradation potential to a predicted degradation rate in aquifers is not possible with our current knowledge.

The biodegradation rate of the two phenoxy acid herbicides, 2,4-D and MCPP have been studied in simple laboratory batch test with groundwater and aquifer sediment under the natural conditions and at low pesticide concentration typically found in the groundwater (**Torang et al., 2003**). In that way the tests intended to mimic the biodegradation of pesticide occurring in aquifers. Below a certain threshold concentration of approximately 1 µg/L for 2,4-D and 10 µg/L for MCPP, the biodegradation followed first order non-growth kinetics and no adaptation was observed within the experimental period (**Torang et al., 2003**). Estimated half-lifes for ultimate degradation in the pristine aquifer with initial concentrations below the threshold concentration were in the order of 500 days for 2,4-D (Figure 16b) and 1100 days for MCPP. 2,4-D was always degraded faster than MCPP. In comparison with these removal rates, an earlier investigation by Larsen et al. (2000) of the vertical distribution of the mineralization potential of MCPP at 25 µg/L have shown half-lifes from 19 days in the topsoil to 370-2600 days at depths from 3.4 to 7.7 m below the surface. Thus, even if there is no adaptation to low concentrations on a larger time scale, biodegradation can be a significant removal process, considering the often much longer retention times that occurs in aquifers than the above estimated range for degradation half-lifes.

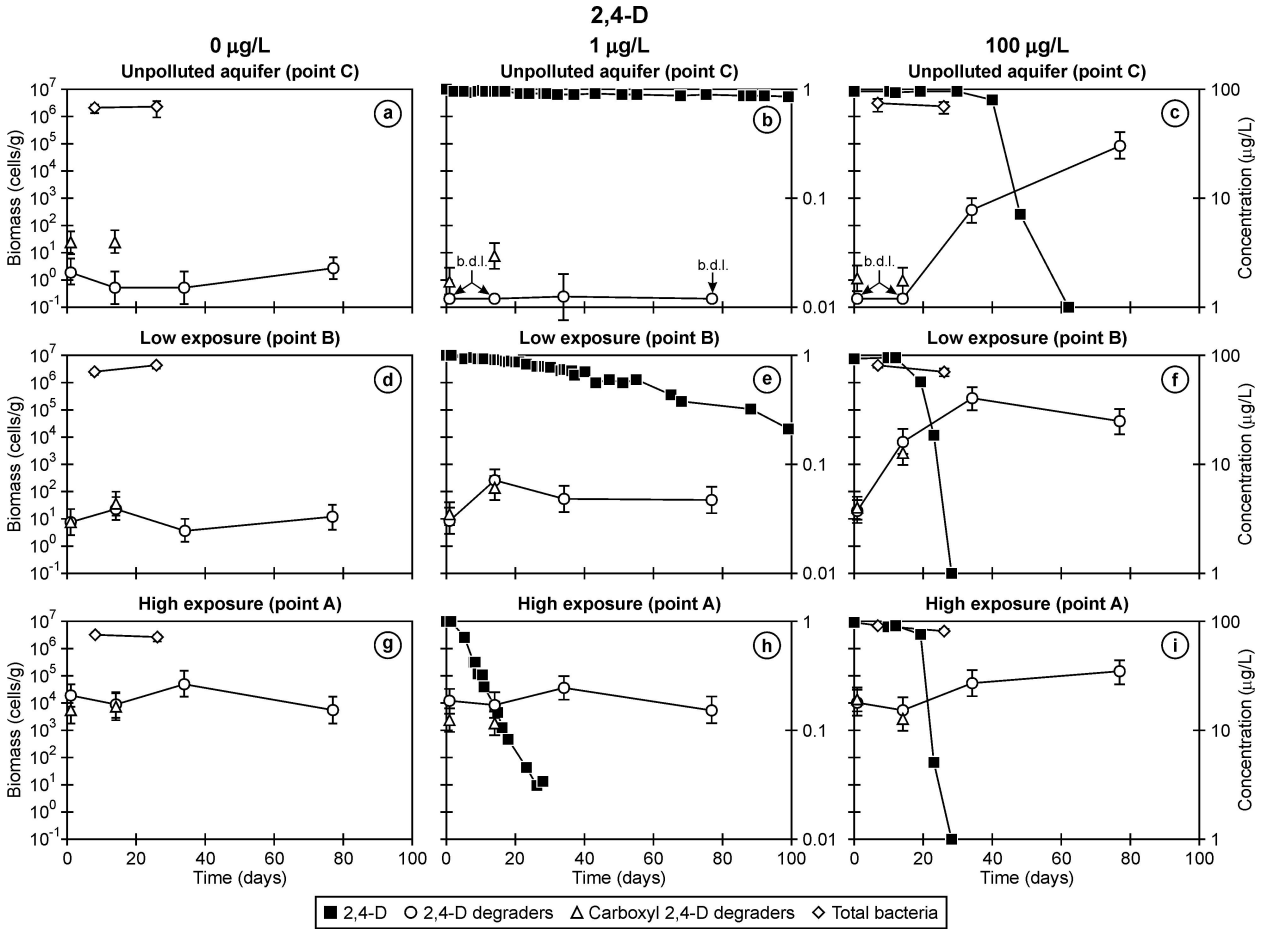

Figure 16. Concentrations of 2,4-D, specific (^{14}C-MPN), and total bacterial counts (AODC-staining) over time, at three concentrations of 2,4-D (0, 1 and 100 µg/L), using aquifer sediment with different in situ exposure history. Experiments were performed at 10°C under aerobic conditions with aquifer sediment from sampling point A (high exposure), B (low exposure), and C (unpolluted). b.d.l. = below detection limit. Error bars for the biomass represent the 95% confidence intervals. From Torang et al., 2003.

Little is known about how long microorganisms remain induced or active in the groundwater environment. This have been studied in laboratory tests adapted to different concentrations where subsamples monthly were respiked to 1 µg/L 2,4-D (**Torang et al., 2004**). Adaptation with enhanced 2,4-D degradation rate was maintained in the batch tests for at least four month but with a slight reduction in the degradation rate over time. This is in agreement with in situ observations following a continuous field injection experiments with exposure to low (~ 40 µg/L) herbicide concentrations (Broholm et al., 2001). Here it was shown that the field aquifer microorganisms were still adapted one year after the end of

exposure (Figure 16) and that the numbers of specific degraders were still in the same range as reported 40 d after the exposure ended (de Lipthay et al., 2003).

Field experiments performed with phenoxy acids below the threshold concentrations (<10 µg/L) have to my knowledge not been performed. However, Broholm et al. (2001) have conducted a continuous, natural gradient, field injection experiment, involving six herbicides and a tracer, in a shallow aerobic aquifer near Vejen, Denmark. The two phenoxy acids MCPP and dichlorprop were injected at concentration of approximately 40 µg/L and were both degraded in the aerobic aquifer. Near the source a lag phase was observed followed by fast degradation of the phenoxy acids, indicating growth kinetics. The phenoxy acids were reported to be completely degraded within 1 m downgradient of the injection wells.

4.3 Methodological and technical limitations

One important goal in a batch simulation test is the estimation of a degradation rates under the actual field conditions which is considered to be representative in a given environmental compartment. "Real World" biodegradation rates refer to the rates at which chemicals biodegrade in surface water, sea, sediment, soil, or ground water after the chemicals has been released from a contamination source e.g., agriculture, landfill, hazardous waste site, waste water effluent, atmospheric depositions (Howard, 1993).

An important limitation in the use of batch simulation tests is that a standardized method may not be available (e.g. groundwater) or that very little experiences have been published about these tests. Therefore further studies and work on standardized methods are strongly needed for the batch simulation tests, but even for the existing methods some general problems have been identified in the interpretation of these tests. These problems are shortly discussed below.

General interpretation problems:

The number of data points in the degradation "window" and the analytical accuracy. This is one of the bottlenecks with batch tests often suffering from the fact that too few data points with to low accuracy were obtained during the degradation time window. Even if samples are taken daily (which may also disturb the test system) this is not frequent enough to determine the precise course of the degradation curve with rapidly biodegradable compounds

such as aniline. This may result in a high degree of uncertainty on the kinetic parameters determined. Because chemical analytical results are obtained delayed after sampling, and because the duration of lag phases can hardly be predicted it is a practical problem of the batch technique to sample frequently enough within the narrow time window where degradation happens to take place after lag phases of variable durations. Reliable parameter estimation requires at least 3-4 data points between some 10-20 % and 80-90 % degradation for an accurate estimation of the lag phase and the degradation rate.

Simplified descriptions of the degradation kinetics. The common use of simple degradation kinetics (e.g. first order or pseudo first order kinetics) may not be appropriate in all test systems. Some test results often with initial concentration above 10 µg/L can much better be described by growth-linked kinetics with accelerating degradation rates where the specific microorganisms proliferate due to the exposure – even at these low µg/L concentrations (**Torang et al., 2003**).

Primary biodegradation versus ultimate degradation. In some tests, only the disappearance of the parent compound (i.e. primary degradation) is determined for example by following the degradation by specific or group specific chemical analyses of the test substance. Data on primary biodegradability may be used for demonstrating rapid degradability, only when it can be satisfactorily demonstrated, that the degradation products formed do not fulfil the criteria for classification as hazardous to the aquatic environment. Ultimate degradation of a compound by microorganisms will result in the degradation to CO_2, H_2O, minerals salts, and new microbial cellular constituents and is often determined by use of ^{14}C-labeled substances. The problem with the use of radiotracer technique is that only the fraction mineralized to CO_2 (either directly or indirectly) is determined. Since organic compounds seldom are complete mineralized and part of the carbon often is assimilated in the biomass, it is not possible to conclude that no parent compound is left in the test system even if no further mineralization products are detected. Combined use of both chemical analyses and radiotracer techniques seems as the most promising tool for interpreting results from batch simulation tests. In that way it is possible to assess whether there is any residual concentration of the parent compound (above the detection limit) and to ensure that the compound is ultimate degraded and not only transformed to a potentially problematic metabolite.

Analytical problems due to the low test concentration. The use of ^{14}C-labelled chemicals and radiotracer technique is many times necessary for sufficiently sensitive analytical measurements to follow the degradation and also allowing the assessment of ultimate degradation rates in contrast to rates of primary degradation. However, these labelled chemicals are often quite expensive if they are available and very expensive if have to be synthesized (approximately 10-20.000 $). The use of specific analysis and measurement of primary biodegradability can also be used if a sufficiently sensitive analytical method is available and information of primary degradation is sufficient. However, these sufficiently sensitive analytical method are many times not available and compromises with use of relatively high concentrations have sometimes to be accepted (see e.g. Ingerslev et al. (2001)).

Small test volumes. Artificial phenomenon and variable test results may arise if batch simulation tests are performed with to small test volumes as discussed by **Ingerslev et al. (2000**). The important message of their study is that when biodegradation tests are designed, test volume should be large enough to prevent occurrence of false negative results. False negative results mean failure of compounds to degrade in the specific test system even though the chemical is generally environmental degradable. This conclusion should be kept in mind when results from biodegradation studies are interpreted.

Variation in simulation test results. As the estimated degradation rates presented in section 4.1 and 4.2 suggest – high variability in the determined degradation half-life of organic chemicals can and must be expected due to differences in environmental conditions as well as differences in the microbial populations not only from place to place but also in time. A number of simulation test data may therefore eventually be available for certain high priority chemicals and will provide a range of half-lives in environmental media such as soil, sediment and/or surface water. The observed differences in half-lives from simulation tests performed on the same substance will reflect differences in test conditions, all of which may be environmentally relevant. A suitable half-life in the higher end of the observed range of half-lives from such investigations should be selected for classification by employing a weight of evidence approach and taking the realism and relevance of the employed tests into account in relation to environmental conditions. In general, simulation test data of surface water should be preferred relative to aquatic sediment or soil simulation test data in relation to the evaluation of the degradability in the aquatic environment.

False negative test results may occur due to the circumstance that the simple batch system used has a limited lifetime and may deteriorate in time and loose its specific degradation ability and/or its similarity with nature. During a batch experiment the diversity of the microbial community may decrease with time due to various loss mechanisms and insufficient selection pressure or unfavorable growth conditions, e.g. due to depletion of the water sample of essential nutrients and primary carbon substrates (Painter, 1995).

In batch simulation tests adaptation must in some way be taken into account and allowed for, because in the real world adaptation is known to play a crucial role (Alexander, 1999). We have therefore suggested a generalised environmentally-realistic semi-continuous preexposure procedure (SCEP) (**Torang and Nyholm, 2005**). The aim is to eliminate the risk of false negative test results and to minimize the problem of variable duration of lag phases and thus optimize sampling schemes. The SCEP mimics the processes' conditions as found in the natural environment and in simulation tests with low chemical concentration and is carried out in practice by periodically renewing part of the test suspension with freshly collected surface water making up the replaced volume of water with test compound to the starting concentration, and in this way maintaining the system characteristics.

Semi-continuous operation may result in adapted microbial populations from which well-defined adapted rate constants, with sufficient number of data points on the degradation curves, can be obtained. Having performed a SCEP, the degradation rate constant can be interpreted as a characteristic of an adapted system and is similar to rate constants obtained in successfully adapted batch systems where long lag phases may be needed. The procedure is believed to be environmentally realistic and it eliminates or reduces lag phases without increasing the subsequent degradation rates other than marginally. SCEP seems to safeguard against loss of specific degradative ability and resulting failure of adaptation or sudden discontinuation of degradation. The semi-continuous preexposure procedure is recommended as an option in biodegradability simulation studies with low chemical concentrations if adaptation takes place slowly or unpredictably. The result produced is an adapted rate and ideally a steady state rate characteristic of a fully adapted test system.

Difficulties in testing mixtures / Multi-component substances. The harmonized criteria for classification of chemicals as hazardous for the aquatic environment focus on single substances. Certain types of intrinsically complex substances (or multi-component substances) typically of natural origin need occasionally to be considered. Such complex chemicals are

normally considered as single substances in a regulatory context. In most cases they are defined as a homologous series of substances within a certain range of carbon chain length and/or degree of substitution. When this is the case, no major difference in degradability is foreseen and the degree of degradability can be established from tests of the complex chemical. However, one exception would be when a borderline degradation is found because in this case some of the individual substances may be rapidly degradable and other may be not rapidly degradable. This requires a more detailed assessment of the degradability of the individual components in the complex substance.

Bioavailability of the substance. Degradation of organic substances in the environment takes place mostly in the aquatic compartments or in aquatic phases in soil or sediment. Moreover, biodegradation requires that the microorganisms are directly in contact with the substance. Addition of highly lipophilic compounds can be very difficult and constitute a serious problem in batch tests. Often an organic solvent is used to add the lipophilic compound but this will still influence the destitution and the bioavailability of the substance. Aging and complexing phenomenon may also significantly affect the bioavailability and thus also the observed degradation rates (Alexander, 1999). Thus the estimated degradation rates may be different from real world biodegradation rates.

5 Gaps in knowledge

Very little experiences have yet been published about the batch simulation tests compared to the vast amount of results from screening tests. More knowledge is required regarding the degradation rate of chemicals in order to focus future work on chemical risk assessment. Hopefully this may eventually lead to the development of a global database (or improvement in existing databases) containing results from all kinds of biodegradation experiments. Information which as a minimum should be available includes the name of test compound, description of test system, test conditions, degradation rate parameters, and literature references.

For future work to improve the understanding of the biodegradation process and for the development of new and/or improved batch simulation tests, I find the following tropics the most important:

- *How will the presence of suspended sediment affect the biodegradation rate?*
Only very limited experiences are available about the effect of suspended sediment in the pelagic surface water test with sediment amendment (e.g. 1 gSS/L). The few existing studies reveal so far that the presence of sediment can both increase and decrease the degradation rate (Ingerslev and Halling-Sorensen, 2001;Ingerslev and Nyholm, 2000;Ingerslev et al., 1998;Nyholm and Torang, 1999).

- *Do primary substrates affect the threshold concentration for growth of specific degraders?*
There seems to be a correlation between low threshold concentrations in environments with low concentration of natural bioavailable substrates (e.g. in groundwater) and vice versa.

- *Can the biodegradation rate of poorly soluble substance be estimated in the existing batch simulation tests?* Here I especially concerned about how well the tests will simulate real world fate with respect to aging, bioavailability and desorption.

- *Can biodegradability and/or degradation rates be predicted?* i) From extrapolation of rates in different environments and / or at different conditions. ii) From improved extrapolation of result from screening tests to degradation rates in different environments. iii) With increased knowledge and improvements of QSAR it may be possible to predict biodegradation rates from chemical structure alone.

- *Can new molecular tools such as different probes make the microbiological characterization easier and allow a quick estimation of the active degrading microorganisms?* This will enable much more reliable test results and make it possible to improve the biodegradation models.

6 Conclusion and perspectives

The study resulted in an increased process understanding of adaptation and of biodegradation of organic compounds in batch simulation tests. The results from the experiments in this thesis and from the literature review clearly demonstrate that direct use of the biodegradation rates obtained in routine degradation studies at high concentrations (> 100 µg/L) may grossly overestimate the actual rates at environmental relevant concentrations, even when investigated in the same environment. This underlines, that for direct interpretation of measured rates it is important to keep the concentrations of simulation studies as low as the actual concentrations, or sufficiently low, to ensure the same kinetics as in the environment.

Biodegradation rates in laboratory systems can be affected by concentration and prior exposure, and adaptation must be considered when such systems are used to predict the fate of xenobiotics in the environment. If biodegradation rate in an adapted environment receiving a continuous exposure is to be assessed this can be achieved by preadaptation of the batch tests. Here a semi-continuous preexposure procedure has been suggested that mimic the conditions in the following batch test including test concentration and other primary substrates.

Further improvement of batch simulation tests performed under laboratory conditions are still necessary if the objective is to obtain an overall rate constant to be used in models for predicting environmental concentration of xenobiotics. There is a pronounced need for increased knowledge about the biodegradation process but the batch simulation tests are considered to be a first step towards a better understanding of biodegradation rates in the environment. Even though there during the last years have been significant improvements of the QSARs and related tools for predicting the biodegradation potential of xenobiotics, there is still a long way before we with a reasonable accuracy can predict the degradation rate in the receiving environment from the chemical structure alone, from the simple screening tests, or transfer rates from one simulation tests to an other environmental compartment and/or other conditions.

Maybe biodegradation will prove as elusive to predict as the weather.

Howard P.H. (1993)

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Appendix 1 - Glossary and abbreviations

In the thesis the words below have been used with the following definitions and are mainly based on definitions accepted by ISO and/or OECD:

Acclimatization	incubated under the same condition as in the test but without the test substance. *Notice* - often used with the same meaning as "adaptation".
Adaptation	the positive result of exposure to the test substance attempting to enhanced degradation rate.
4-ClA	4-chloroaniline
2,4-D	2,4-dichlorphenoxyacetic acid
Exposure	A test system or a given environment is influenced by a specific or structural related compounds often resulting in an adaptation of the microorganisms.
False negative results	Failure to degrade compounds in a specific test system even though the chemical is generally environmental degradable
First order degradation rate constant	A first order or pseudo first order kinetic rate constant, k (d^{-1}), which characterizes the rate of the degradation processes. For a batch experiment k is estimated from the initial part of the degradation curve obtained after the end of the lag phase.
Half-life, $T_{½}$	Term used to characterize the rate of a first order reaction. It is the time interval that corresponds to a concentration decrease

	by a factor 2. The half-life and the degradation rate constant are related by the equation $T_{½} = \ln 2 / k$.
HPV	High production volume (substances produced annually in volumes of more than 1,000 tonne).
Lag phase	The time from the start of a test until adaptation of the degrading micro-organisms is achieved and the biodegradation degree of a chemical substance or organic matter has increased to a detectable level (e.g. 10 % of the maximum theoretical biodegradation, or lower, dependent on the accuracy of the measuring technique).
MCPP	Mecoprop, (+/-)-2-(4-chloro-2-methylphenoxy) propanoic acid
Mineralization (aerobic)	the test substance is fully oxidized resulting in the production to CO_2, H_2O and minerals salts.
PBTs	Substances of very high concern that are persistent (difficult to break down), bio-accumulative (accumulate in our bodies) and toxic.
Persistent	test substance is not degradable in any environmental compartment under environmentally realistic conditions.
PNP	4-nitrophenol
Primary biodegradation	The structural change (transformation) of a chemical substance by microorganisms resulting in the loss of chemical identity.
Primary substrate	A collection of natural carbon and energy sources that provide growth and maintenance of the microbial biomass (adopted after (Rittmann, 1992).

Secondary substrate	A substrate component present in a such low concentration, that by its degradation, only insignificant amounts of carbon and energy are supplied to the competent micro-organisms, as compared to the carbon and energy supplied by their degradation of main substrate components (primary substrates) (adopted after (Rittmann, 1992).
TGD	Technical Guidance Document from the European Commission
Ultimate biodegradation (aerobic)	the test substance is totally utilized by microorganisms resulting in the production to CO_2, H_2O, minerals salts, and new microbial cellular constituents (biomass).
Xenobiotic	Manmade chemicals not produced by any known plants, animals or microorganisms.

Appendix 2 - TEST GUIDELINES

Most of the guidelines mentioned below are found in compilations from the organization issuing them. The main references to these are:

- OECD guidelines for the testing of chemicals. OECD, Paris, 1998 with regular updates (Homepage: http://www.oecd.org/env/testguidelines);
- EC guidelines: European Commission (2003). Consultation document concerning REACH. European Commission, Brussels (Homepage: http://europa.eu.int/comm/enterprise/chemicals/chempol/reach/volume5.pdf);
- ISO guidelines: Available from the national standardization organizations or ISO (Homepage: http://www.iso.ch/). An overview on exciting ISO biodegradation tests is given in ISO 15462.
- Office of Pollution Prevention & Toxics Substances (OPPTS). Guidelines from the US-EPA (Homepage: http://www.epa.gov/opptsfrs/home/guidelin.htm);
- American Society for Testing and Materials (ASTM): ASTM´s homepage: http://www.astm.org. Further search via "standards".

ASTM E1279-89 (2001) Standard test method for biodegradation by a shake-flask die-away method

ASTM E1625-94 (2001) Standard Test Method for Determining Biodegradability of Organic Chemicals in Semi-Continuous Activated Sludge (SCAS)

EC C.4. Determination of ready biodegradability. Directive 92/69/EEC

EC C.5. Degradation: biochemical oxygen demand. Directive 92/69/EEC

EC C.9. Biodegradation: Zahn-Wellens test. Directive 92/69/EEC

EC C.10. Biodegradation: Activated sludge simulation tests. Directive 92/69/EEC

EC C.11. Biodegradation: Activated sludge respiration inhibition test. Directive 92/69/EEC

EC C.12. Biodegradation: Modified SCAS test. Directive 92/69/EEC

ISO 7827 (1994) Water quality - Evaluation in an aqueous medium of the "ultimate" aerobic biodegradability of organic compounds - Method by analysis of dissolved organic carbon (DOC)

ISO 9408 (1999) Water quality - Evaluation in an aqueous medium of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer

ISO 9439 (1999) Water quality - Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium - Carbon dioxide evolution test

ISO 9887 (1992) Water quality - Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium – Semi-continuous activated sludge method (SCAS)

ISO 9888 (1999) Water quality - Evaluation of the ultimate aerobic biodegradability of organic compounds in an aqueous medium - Static test (Zahn-Wellens method)

ISO 10634 (1995) Water quality - Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium

ISO 10707 (1994) Water quality - Evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic compounds - Method by analysis of biochemical oxygen demand (closed bottle test)

ISO 10708 (1997) Water quality - Evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic compounds - Method by determining the biochemical oxygen demand in a two-phase closed bottle test

ISO 11733 (2002) Water quality - Evaluation of the elimination and the biodegradability of organic compounds in an aqueous medium - Activated sludge simulation test

ISO 14592-1 (2002) Water quality - Evaluation of the aerobic biodegradability of organic compounds at low concentrations. Part 1: Shake flask batch test with surface water or surface water/ sediment suspensions

ISO 14592-2 (2002) Water quality - Evaluation of the aerobic biodegradability of organic compounds at low concentrations. Part 2: Continuous flow river model with attached biomass

ISO 14593 (1999) Water quality - Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium - Method by analysis of inorganic carbon in sealed vessels (CO_2 headspace test)

ISO 15462 (1997) Water quality - Selection of tests for biodegradability (Technical Report)

ISO 16221 (2001) Water quality - Guidance for determination of biodegradability in the marine environment

OECD Test Guideline 301 (1992). Ready biodegradability. OECD guidelines for testing of chemicals

OECD Test Guideline 302A (1981). Inherent biodegradability: Modified SCAS test. OECD guidelines for testing of chemicals

OECD Test Guideline 302B (1992). Zahn-Wellens/EMPA test. OECD guidelines for testing of chemicals

OECD Test Guideline 302C (1981). Inherent biodegradability: Modified MITI test (II). OECD guidelines for testing of chemicals

OECD Test Guideline 303A (1981). Simulation test - aerobic sewage treatment: Coupled units test. OECD guidelines for testing of chemicals. Draft update available 1999

OECD Test Guideline 304A (1981). Inherent biodegradability in soil. OECD guidelines for testing of chemicals

OECD Test Guideline 306 (1992). Biodegradability in seawater. OECD guidelines for testing of chemicals

OECD Test Guideline 307 (2002) Aerobic and Anaerobic Transformation in Soil. OECD guidelines for testing of chemicals

OECD Test Guideline 308 (2002) Aerobic and Anaerobic Transformation in Aquatic Sediment Systems. OECD guidelines for testing of chemicals

OECD 309 (2002). Simulation test - Aerobic Transformation in Surface Water. Draft proposal for a new guideline, TG 309 February/March 2002.

OECD 310 (2002) Ready Biodegradability - CO2 in Sealed Vessels (Headspace Test). Draft Test Guideline 310. OECD guidelines for testing of chemicals

835.3100 Aerobic aquatic biodegradation

OPPTS 835.3110 Ready biodegradability

OPPTS 835.3120 Sealed-vessel carbon dioxide production test

OPPTS 835.3160 Biodegradability in sea water

OPPTS 835.3170 Shake flask die-away test

OPPTS 835.3180 Sediment/water microcosm biodegradation test

OPPTS 835.3200 Zahn-Wellens/EMPA test

OPPTS 835.3210 Modified SCAS test

OPPTS 835.3300 Soil biodegradation

OPPTS 835.5045 Modified SCAS test for insoluble and volatile chemicals